# CONJUGATES OF SOLUBLE PEPTIDIC COMPOUNDS WITH MEMBRANE-BINDING AGENTS

This invention relates to polypeptide derivatives, their use in therapy and methods and intermediates for their production.

Essentially all protein drugs are administered as solutions and function *in vivo* in the solution phase. In biochemistry and pharmacology, however, a large number of control and mediator proteins are associated with or function within or on the plasma membranes of cells. Except for soluble, truncated versions of one class of these molecules, no membrane-associated proteins have been developed as therapeutic agents. There are two main reasons for this situation. Firstly, overexpression of proteins that are retained in the membranes of the producer cells is limited by the the low capacity of membranes for proteins and often by the toxic effects of retention when expression is intrinsically efficient. Secondly, extraction of these proteins from membranes requires detergents or organic solvents, often results in inactivation of the protein, leads to difficulties in achieving the high purity needed for drug use and usually gives a product which is hard to formulate for intravenous administration. In addition, retention of very hydrophobic membrane anchoring elements may cause proteins to associate strongly with lipid-binding proteins in blood when administered intravenously thus preventing access to cell membranes.

Soluble, truncated versions of membrane-associated proteins overcome the production difficulties associated with full length proteins. However such truncated molecules lack the membrane binding capability and specificity of the full length proteins which properties may be advantageous or even essential to the desired therapeutic activity.

The main classes of interaction of proteins with membranes can be summarised as follows:

1. Direct and specific interactions with phospholipid head groups or with other hydrophilic regions of complex lipids or indirectly with proteins already inserted in the membrane. The latter may include all the types of intrinsic membrane protein noted below and such interactions are usually with extracellular domains or sequence loops of the membrane proteins;

2. Through anchoring by a single hydrophobic transmembrane helical region near the terminus of the protein. These regions commonly present a hydrophobic face around the entire circumference of the helix cylinder and transfer of this structure to the hydrophilic environment of bulk water is energetically unfavourable.

3. Further binding is provided [illegible] ionic aminoacids [illegible] cytoplasmic side [illegible] membrane [illegible] transmembrane helix.

4. Through the use of multiple (normally 2-12 and commonly 4,7 and 10) transmembrane regions which are usually predicted to be helical or near-helical. Although these regions are normally hydrophobic overall, they frequently show some amphipathic behaviour - an outer hydrophobic face and an inner more hydrophilic one being identifiable within a helix bundle located in the lipid bilayer;

5. Through postranslationally linked phosphatidyl inositol moeities (GPI-anchors). These are generated by a specific biosynthetic pathway which recognises and removes a specific stretch of C-terminal aminoacids and creates a membrane-associating diacyl glycerol unit linked *via* a hydrophilic carbohydrate spacer to the polypeptide;

6. In a related process, single fatty acid groups such as myristoyl, palmitoyl or prenyl may be attached postranslationally to one or more sites in a protein (usually at N- or C-termini). Again, amino acids (such as the C-terminal CAAX box in *Ras* proteins) may be removed.

Artificial membranes are considered to be lipid complexes that mimic the basic properties of the cell membrane, i.e., a lipid vacuole with an aqueous interior and a surface chemistry that resembles the cell membrane. The artificial membrane typically contains phospholipids or mimics thereof and may be unilemellar or bilemellar and the outer surface will contain charged groups similar to the choline groups of the most abundant phospholipid. The prototype artificial membrane is known as a liposome and the technologies for the construction of liposomes including the incorporation of therapeutically useful agents into them is well known to those in the art. Liposomes have been evaluated in a number of disease states and liposomes containing the anti-fungal Amphotericin are commercially available. In addition, proteoliposomes have been described. For example, the use of immunoliposomes encapsulating amphotericin B has been reported to be of benefit in the treatment of experimental fungal infections in animal models (e.g. Hospenthal, D. et al (1989) J. Med. Microbiol. **30** 193-197; Dromer, F. et al (1990) Antimicrob. Agents Chemother. **34** 2055-2060).

Mimics of natural or artificial membranes are often related in structure and will mimic one or more properties of the membrane. One such example is the provision of an artificial surface having pendant groups which mimic the phospholipid zwitterionic groups which are found on the outside of cell surfaces. For example WO92/06719 (Biocompatibles Limited) discloses natural and synthetic phospholipids which may be coated on an artificial surface, e.g. a device which, in use, will come into contact with

[illegible] biocompatibility and WO 94/16749 discloses additional zwitterionic groups that may be used to improve biocompatibility in a similar way.

The present invention provides a soluble derivative of a soluble polypeptide, said derivative comprising two or more heterologous membrane binding elements with low membrane affinity covalently associated with the polypeptide which elements are capable of interacting, independently and with thermodynamic additivity, with components of cellular or artificial membranes exposed to extracellular fluids.

By 'heterologous' is meant that the elements are not found in the native full length protein from which a soluble protein may be derived.

By 'soluble polypeptide' is meant a truncated derivative of a full length protein which lacks its natural membrane binding capability, and/or a polypeptide which has a solubility level in aqueous media of > 100µg/ml.

By 'membrane binding element with low membrane affinity' is meant that the element has only moderate affinity for membranes, that is a dissociation constant greater than 0.1µM, preferably 1µM-1mM. The elements preferably have a size <5kDa.

The derivative should incorporate sufficient elements with low affinities for membrane components to result in a derivative with a high (preferably 0.01 - 10nM dissociation constant) affinity for specific membranes. The elements combine so as to create an overall high affinity for the particular target membrane but the combination lacks such high affinity for other proteins for which single elements may be (low-affinity) ligands.

The elements should be chosen so as to retain useful solubility in pharmaceutial formulation media, preferably >100µg/ml. Preferably at least one element is hydrophilic.

The invention thus promotes localisation of a therapeutic protein at cellular membranes and thereby provides one or more of several biologically significant effects with potential therapeutic advantages including:

Potency: If the protein is a receptor and an agonist or antagonist activity is localised on the same surface as the receptor itself, an increase in effective concentration may result from the reduction in the diffusional degrees of freedom.

Pharmacokinetics and dosing frequency: Interaction of a derivatised protein with long-lived cell types or serum proteins would be expected to prolong the plasma residence time of the protein and produce a depot effect through deposition on cell surfaces.

Specificity: Many clinically important pathological processes are associated

[illegible] below). Hence targeting the modified protein to regions of membrane containing

pathology-associated membrane markers may improve the therapeutic ratio of the protein targeted.

The derivatives of the invention may be used in association with artificial membranes or mimics thereof to allow delivery of the therapeutic protein to sites where it will provide therapeutic benefit. For example, polypeptides associated with liposomes formed by contacting liposomes with a derivative of the invention may be more stable than the free polypeptide. The liposome may incorporate a therapeutic agent, for example an antiflammatory or cytotoxic agent. The polypeptide derivative of the invention may thus be used to target the therapeutic agent. When the polypeptide is itself a therapeutic agent, the liposome incorporated therapeutic agent may be used to enhance further the efficacy or tolerability of the therapy.

Association of derivatives of the invention with mimics of cell membranes may be used to concentrate the therapeutic protein at sites where therapeutically useful concentrations of underivatised protein might be difficult to achieve. For example, indwelling medical devices coated with mimics of the phospholipid zwitterionic groups which are found on the outside of cell surfaces, such as those disclosed in WO92/06719 and WO 94/16749, may be additionally treated with derivatives of the invention. For example complement inhibitors derivatised in accordance with the invention could be incorporated into the outer surface of indwelling catheters or hip replacements or heart valves in order to minimise development of inflammation associated with these operations.

It will be appreciated that all associations of heterologous amino acid sequences with a polypeptide which is a soluble derivative of a human protein will need to be assessed for potential immunogenicity, particularly where the amino acid sequence is not derived from a human protein. The problem can be minimised by using sequences as close as possible to known human ones and through computation of secondary structure and antigenicity indices.

Examples of protein therapeutic agents which may be modified according to the invention include but are not restricted to the following:

| Base Protein | Cell Target | Therapeutic Application |
|---|---|---|
| IL-4 Y124D mutein | B-cells | Anti-allergy (IL-4 antagonist) |
| Plasminogen activators e.g. Prourokinase, streptokinase, tissue-type plasminogen activator, reteplase | Erythrocytes, vascular endothelium | Prevention of venous thrombosis |
| Leptin | Choroid plexus, Hypothalamus | Weight loss (agonist) |
| Complement inhibitors* | Vascular endothelium, Myocytes, Erythrocytes, Lymphocytes | Ischaemic injury, transplantation, inflammation |
| scFv antibody against cytokines (IL-1, IL-, IL-5, IL-6) | Eosinophils | Asthma, allergic disease |
| Protein C | Vascular endothelium | Prevention of venous thrombosis |
| Antibodies against CD4, B7/CD28, CD3/TCR, CD11b(CR3) | Lymphocytes | Immunosuppression |
| Interferon-β and derivatives | Lymphocytes | Immunomodulation, multiple sclerosis |

*Complement regulatory proteins e.g.: CR1 (CD35); DAF (CD55); MCP (CD46); CD59; Factor H; and C4 binding protein; and hybrids or muteins thereof such as CR1-CD59 (S.G.El Feki and D.T.Fearon Molecular Immunology **33** (supp 1). p 57, 1996), MCP-DAF (P.J.Higgins *et al*, J.Immunology. **158**, 2872-2881,1997) and soluble CR1 polypeptide fragments.

The derivative preferably comprises two to eight, more preferably two to four membrane binding elements.

Membrane binding elements are preferably selected from: fatty acid derivatives such as fatty acyl groups; basic amino acid sequences; ligands of known integral membrane proteins; sequences derived from the complementarity-determining region of monoclonal antibodies raised against epitopes of membrane proteins; membrane binding sequences identified through screening of random chemical or peptide libraries

components thereof

Suitable fatty acid derivatives include myristoyl (12 methylene units) which is insufficiently large or hydrophobic to permit high affinity binding to membranes. Studies with myristoylated peptides (eg R.M.Peitzsch & S.McLaughlin, Biochemistry, 32, 10436-10443, 1993)) have shown that they have effective dissociation constants with model lipid systems of ~$10^{-4}$ M and around 10 of the 12 methylene groups are buried in the lipid bilayer. Thus, aliphatic acyl groups with about 8 to 18 methylene units, preferably 10-14, are suitable membrane binding elements. Other examples of suitable fatty acid derivatives include long-chain (8-18, preferably 10-14 methylene) aliphatic amines and thiols, steroid and farnesyl derivatives.

Membrane binding has been found to be associated with limited (single-site) modification with fatty acyl groups when combined with a cluster of basic aminoacids in the protein sequence which may interact with acidic phospholipid head groups and provide the additional energy to target membrane binding. This combination of effects has been termed the 'myristoyl-electrostatic switch' (S.McLaughlin and A.Aderem, TIBS, 20,272-276, 1994; J.F.Hancock *et al*, Cell, 63, 133-139,1990). Thus, a further example of suitable membrane binding elements are basic aminoacid sequences such as those found in proteins such as *Ras* and MARCKS (myristoylated alanine-rich C-kinase substrate, P.J. Blackshear, J. Biol. Chem., 268, 1501-1504, 1993) which mediate the electrostatic 'switch' through reversible phosphorylation of serine residues within the sequence and a concomitant neutralisation of the net positive charge. Such sequences include but are not restricted to consecutive sequences of Lysine and Arginine such as (Lys)n where n is from 3 to 10, preferably 4 to 7.

Suitable examples of amino acid sequences comprising basic amino acids include:

i) DGPKKKKKKSPSKSSG

ii) GSSKSPSKKKKKKPGD

iii) SPSNETPKKKKKRFSFKKSG

iv) DGPKKKKKKSPSKSSK

v) SKDGKKKKKKSKTK

(N-terminus on left)

Sequences i) to v) are examples of electrostatic switch sequences.

Examples of amino acid sequences derived from ligands of known integral membrane proteins include RGD-containing peptides such as GRGDSP which are ligands for the $\alpha_{IIb}\beta_3$ integrin of human platelet membranes. Another example is DGPSEILRGDFSS derived from human fibrinogen alpha chain, which binds to the GpIIb/IIIa membrane protein in platelet

interactions between membrane proteins such as receptors and the major

histocompatibility complex. An example of such a membrane protein ligand is the sequence GNEQSFRVDLRTLLRYA which has been shown to bind to the major histocompatibility complex class 1 protein (MHC-1) with moderate affinity (L.Olsson *et al*, Proc. Natl .Acad.Sci.USA. 91, 9086-909, 1994).

Yet further examples of such sequences employ a membrane insertive address specific for T-cells. Such sequence is derived from the known interaction of the transmembrane helix of the T-cell antigen receptor with CD3 (Nature Medicine 3, 84-88,1997). Examples are peptides containing the sequence GFRILLLKV such as:

SAAPSSGFRILLLKV

AAPSVIGFRILLLKVAG

An example of a ligand for an integral membrane protein is the carbohydrate ligand Sialyl Lewis$^x$ which has been identified as a ligand for the integral membrane protein ELAM-1 (M.L.Phillips *et al*, Science, 250, 1130-1132, 1990 & G.Walz *et al*, *Ibid*, 250, 1132-1135,1990).

Sequences derived from the complementarity-determining regions of monoclonal antibodies raised against epitopes within membrane proteins (see, for example, J.W.Smith *et al*, J.Biol.Chem. 270, 30486-30490, 1995) are also suitable membrane binding elements, as are binding sequences from random chemical libraries such as those generated in a phage display format and selected by biopanning operations *in vitro* (G.F.Smith and J.K.Scott, Methods in Enzymology, 217H, 228-257,1993) or *in vivo* (R.Pasqualini & E.Ruoslahti, Nature, 380, 364-366, 1996).

Optionally, conditional dissociation from the membrane may be incorporated into derivatives of the invention using mechanisms such as pH sensitivity (electrostatic switches), regulation through metal ion binding (using endogenous $Ca^{2+}$, $Zn^{2+}$ and incorporation of ion binding sites in membrane binding elements) and protease cleavage (e.g plasminolysis of lysine-rich membrane binding sequences to release and activate prourokinase)

Preferred derivatives of this invention have the following structure:

**[P]-{L-[W]}$_n$-X**

in which:

**P** is the soluble polypeptide,

each **L** is independently a flexible linker group,

each **W** is independently a peptidic membrane binding element,

Peptidic membrane binding elements are preferably 8 to 20 amino acids long and elements **W** are preferably located sequentially either at the N or C terminus of the soluble polypeptide. The amino acid sequences are linked to one another and to the soluble peptide by linker groups which are preferably selected from hydrophilic and/or flexible aminoacid sequences of 4 to 20 aminoacids; linear hydrophilic synthetic polymers; and chemical bridging groups.

Peptide linkages may be made chemically or biosynthetically by expression of appropriate coding DNA sequences. Non peptide linkages may be made chemically or enzymatically by post-translational modification.

The polypeptide portion of the derivatives of the invention may be prepared by expression in suitable hosts of modified genes encoding the soluble polypeptide of interest plus one or more peptidic membrane binding elements and optional residues such as cysteine to introduce linking groups to facilitate post translational derivatisation with additional membrane binding elements.

In a further aspect, therefore, the invention provides a process for preparing a derivative according to the invention which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to chemically introduce membrane binding elements.

In particular, the recombinant aspect of the process may comprise the steps of:

i) preparing a replicable expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said polypeptide portion;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said polypeptide; and

iv) recovering said polypeptide.

Where the polypeptide portion is novel, the DNA polymer comprising a nucleotide sequence that encodes the polypeptide portion as well as the polypeptide portion itself and S-derivatives thereof, also form part of the invention. In particular the invention provides a polypeptide portion of a derivative of the invention comprising the soluble peptide linked by a peptide bond to one peptidic membrane binding element and/or including a C-terminal cysteine, and DNA polymers encoding the polypeptide portion.

The recombinant process of the invention may be performed by conventional

laboratory manual 2nd Edition. Cold Spring Harbor Laboratory Press (1989) and DNA Cloning vols I, II and III (D. M. Glover ed., IRL Press Ltd).

The invention also provides a process for preparing the DNA polymer by the condensation of appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, *in vitro* or *in vivo* as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts *et al.*, in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase 1 (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to 37°C, generally in a volume of 50µl or less.

The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J.Gait, H.W.D. Matthes M. Singh, B.S. Sproat and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al.*, Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes *et al.*, EMBO Journal, 1984, 3, 801. Preferably an automated DNA synthesiser (for example, Applied Biosystems 381A Synthesiser) is employed.

The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA [illegible]

The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the polypeptide is a routine matter for the skilled worker in the art.

In particular, consideration may be given to the codon usage of the particular host cell. The codons may be optimised for high level expression in *E. coli* using the principles set out in Devereux *et al.*, (1984) Nucl. Acid Res., **12**, 387.

The expression of the DNA polymer encoding the polypeptide in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. Novel expression vectors also form part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the polypeptide, under ligating conditions.

The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired. The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as *E. coli*, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary, fungi e.g. filamentous fungi or unicellular 'yeast' or an insect cell such as Drosophila. The host cell may also be in a transgenic animal. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses derived from, for example, baculoviruses or vaccinia.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the fragment e.g. bovine papillomavirus vectors in mouse C127 cells, or amplified vectors in chinese hamster ovary cells (DNA Cloning Vol. II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. *et al.*. Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H. Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. *et al.*,European Patent Application No. 0093619, 1983).

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation

Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Sambrook *et al.*, cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E.coli*, may be treated with a solution of $CaCl_2$ (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol or by electroporation as for example described by Bio-Rad Laboratories, Richmond, California, USA, manufacturers of an electroporator. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells or by using cationic liposomes.

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Sambrook *et al.*, and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The protein product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial such as *E. coli* and the protein is expressed intracellularly, it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product is usually isolated from the nutrient medium.

Where the host cell is bacterial, such as *E. coli*, the product obtained from the culture may require folding for optimum functional activity. This is most likely if the protein is expressed as inclusion bodies. There are a number of aspects of the isolation and folding process that are regarded as important. In particular, the polypeptide is preferably partially purified before folding, in order to minimise formation of aggregates with contaminating proteins and minimise misfolding of the polypeptide. Thus, the removal of contaminating *E. coli* proteins by specifically isolating the inclusion bodies and the subsequent additional purification prior to folding are important [illegible]

The folding process is carried out in such a way as to minimise aggregation of intermediate-folded states of the polypeptide. Thus, careful consideration needs to be given to, among others, the salt type and concentration, temperature, protein concentration, redox buffer concentrations and duration of folding. The exact condition for any given polypeptide generally cannot be predicted and must be determined by experiment.

There are numerous methods available for the folding of proteins from inclusion bodies and these are known to the skilled worker in this field. The methods generally involve breaking all the disulphide bonds in the inclusion body, for example with 50mM 2-mercaptoethanol, in the presence of a high concentration of denaturant such as 8M urea or 6M guanidine hydrochloride. The next step is to remove these agents to allow folding of the proteins to occur. Formation of the disulphide bridges requires an oxidising environment and this may be provided in a number of ways, for example by air, or by incorporating a suitable redox system, for example a mixture of reduced and oxidised glutathione.

Preferably, the inclusion body is solubilised using 8M urea, in the presence of mercaptoethanol, and protein is folded, after initial removal of contaminating proteins, by addition of cold buffer. Suitable buffers may be identified using the techniques described in I.Dodd *et al,* 'Perspectives in Protein Engineering and Complementary Technologies' , Mayflower Publications, 66-69, 1995. A suitable buffer for many of the SCR constructs described herein is 20mM ethanolamine containing 1mM reduced glutathione and 0.5mM oxidised glutathione. The folding is preferably carried out at a temperature in the range 1 to 5°C over a period of 1 to 4 days.

If any precipitation or aggregation is observed, the aggregated protein can be removed in a number of ways, for example by centrifugation or by treatment with precipitants such as ammonium sulphate. Where either of these procedures are adopted, monomeric polypeptide is the major soluble product.

If the bacterial cell secretes the protein, folding is not usually necessary.

The polypeptide portion of the derivative of the invention may include a C-terminal cysteine to facilitate post translational modification. A soluble polypeptide including a C-terminal cysteine also forms part of the invention. Expression in a bacterial system is preferred for proteins of moderate size (up to ~70kDa) and with <~8 disulphide bridges. More complex proteins for which a free terminal Cys could cause refolding or stability problems may require stable expression in mammalian cell lines (especially CHO). This will also be needed if a carbohydrate membrane binding element is to be introduced post-translationally. The use of insect cells infected with [illegible]

preparing more complex proteins and will be preferred when it is desired to carry out certain post-translational processes (such as palmitoylation) biosynthetically (see for example, M.J.Page *et al* J.Biol.Chem. 264, 19147-19154, 1989)

A preferred method of handling proteins C-terminally derivatised with cysteine is as a mixed disulphide with mercaptoethanol or glutathione or as the 2-nitro, 5-carboxyphenyl thio- derivative as generally described below in Methods.

Peptide membrane binding elements may be prepared using standard solid state synthesis such as the Merrifield method and this method can be adapted to incorporate required non-peptide membrane binding elements such as N-acyl groups derived from myristic or palmitic acids at the N terminus of the peptide. In addition activation of an amino acid residue for subsequent linkage to a protein can be achieved during chemical synthesis of such membrane binding elements. Examples of such activations include formation of the mixed 2-pyridyl disulphide with a cysteine thiol or incorporation of an N-haloacetyl group. Both of these groups are capable of reaction with free thiols, through disulphide interchange and alkylation, respectively. Peptides can optionally be prepared as the C-terminal amide and/or with a conventional N-terminal blocking group such as acetyl.

The invention also provides a peptidic membrane binding element comprising one or more derivatisations selected from:

a terminal cysteine residue optionally activated at the thiol group;

an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the the peptide or at an ε-amino group of a lysine residue;

an amide group at the C-terminus;

an N-terminal blocking group; and

a fatty acid N-acyl group at the N-terminus or at an ε-amino group of a lysine residue.

Chemical bridging groups and reagents suitable for their formation include those described in EP0109653, EP0152736, EP0155388 and EP0284413, incorporated herein by reference. The bridging group is generally of the formula:

-A-R-B- (I)

in which each of A and B, which may be the same or different, represents -CO-, $-C(=NH_2^+)-$, maleimido, -S- or a bond and R is a bond or a linking group containing one or more $-(CH_2)-$ or meta-, ortho- or para- disubstituted phenyl units, preferably ortho or para, optionally together with a hydrophilic portion.

Where the polypeptide portion of the derivative of the invention and a peptidic

exchange chemistry, by activating a thiol on one polypeptide and reacting the activated thiol with a free thiol on the other polypeptide. Such activation procedures make use of disulphides which form stable thiolate anions upon cleavage of the S-S linkage and include reagents such as 2,2' dithiopyridine and 5,5'-dithio(2-nitrobenzoic acid, DTNB) which form intermediate mixed disulphides capable of further reaction with thiols to give stable disulphide linkages.

R may include moieties which interact with water to maintain the water solubility of the linkage and suitable moieties include -CO-NH-, -CO-NMe-, -S-S-, -CH(OH)-, $-SO_2-$, $-CO_2-$, $-(CH_2CH_2-O)_m-$ and -CH(COOH)- where m is an integer of 2 or more, or linear hydrophilic polymers such as polyethylene glycol, polypropylene glycol, polyglycine, polyalanine or polysarcosine.

Other examples of R include $-(CH_2)_r-$, $-(CH_2)_p-S-S-(CH_2)_q-$ and $-(CH_2)_p-CH(OH)-CH(OH)-(CH_2)_q-$, in which r is an integer of at least 2, preferably at least 4 and p and q are independently integers of at least 2

In a further aspect R may take the form -U-V-W- where U is $(CH_2)_2CONH(CH_2)_n$ in which n is an integer of 3 to 8, V is O, S, $NR_a$ or $NR_a-NR_a$ where each $R_a$ is H or $C_{1-6}$ alkyl, NH-O or O-NH, and W is benzyl substituted at the 2- or 4- position by the group B. In a preferred embodiment R is $(CH_2)_2CONH(CH_2)_nNH$-(4-phenyl) where n is an integer of 3 to 8.The bridging group of formula (I) may be derived from a linking agent of formula (II):

$$X-R_1-Y \qquad (II)$$

in which $R_1$ is a bond or a linking group and X and Y are functional groups reactable with surface amino acid groups, preferably a lysine or cysteine group, the N-terminal amino group, a catalytic serine hydroxyl or a protein attachment group, and X, $R_1$- and Y are chosen so as to generate the required bridging group -A-R-B-.

Preferred agents are those where X and Y are different, known as heterobifunctional agents. Each end of the agent molecule is reacted in turn with each polypeptide to be linked in separate reactions. Examples of heterobifunctional agents of formula (II) include:

N-succinimidyl 3-(2-pyridyldithio) propionate

succinimidyl 4-(N-maleimido) caproate

3-(2-pyridyl) methyl propionimidate hydrochloride

4'-amidinophenyl 4-N-[2-N-(3-[2-pyridyldithio]ethylcarbonyl)aminoethyl] aminobenzoate hydrochloride.

Other suitable agents are disclosed in EP0109653, EP0152736, EP0155388 and EP0284413, in particular those of formula (II) in EP0155388 and (III) in EP0284413

In each case Y is capable of reacting with a thiol group on a polypeptide, which may be a native thiol or one introduced as a protein attachment group.

The protein attachment group is a functionality derived by modification of a polypeptide or protein with a reagent specific for one or more amino acid side chains, and which contains a group capable of reacting with a cleavable section on the other polypeptide. An example of a protein attachment group is a thiol group. An example of a cleavable section is a disulphide bond. Alternatively the cleavable section may comprise an $\alpha$, $\beta$ dihydroxy function.

As an example, the introduction of a free thiol function by reaction of a polypeptide with 2-iminothiolane, N-succinimidyl 3-(2-pyridyldithio) propionate (with subsequent reduction) or N-acetyl homocysteine thiolactone will permit coupling of the protein attachment group with a thiol-reactive Y structure. Alternatively, the protein attachment group can contain a thiol-reactive entity such as the 6-maleimidohexyl group or a 2-pyridyl-dithio group which can react with a free thiol in X. Preferably, the protein attachment group is derived from protein modifying agents such as 2-iminothiolane that react with lysine $\epsilon$-amino groups in proteins.

When X represents a group capable of reacting directly with the amino acid side chain of a protein, it is preferably an N-succinimidyl group. When X represents a group capable of reacting with a protein attachment group, it is preferably a pyridylthio group. When X represents a group capable of reacting with a catalytic serine hydroxyl it is preferably an 4-amidinophenyl ester group optionally substituted by one or more electron withdrawing groups which increases the reactivity of the ester, of the kind contained in the compounds of formula (II) in EP0155388 and (III) in EP0284413.

In the above processes, modification of a polypeptide to introduce a protein attachment group is preferably carried out in aqueous buffered media at a pH between 3.0 and 9.0 depending on the reagent used. For a preferred reagent, 2-iminothiolane, the pH is preferably 6.5-8.5. The concentration of polypeptide is preferably high (> 10mg/ml) and the modifying reagent is used in a moderate (1.1- to 5-fold) molar excess, depending on the reactivity of the reagent. The temperature and duration of reaction are preferably in the range $0^{0}$-$40^{0}$C and 10 minutes to 7 days. The extent of modification of the polypeptide may be determined by assaying for attachment groups introduced.

Such assays may be standard protein chemical techniques such as titration with 5,5'-dithiobis-(2-nitrobenzoic acid). Preferably, 0.5-3.0 moles of protein attachment group will be introduced on average per mole of polypeptide. The modified polypeptide may be separated from excess modifying agents by standard techniques such as dialysis, diafiltration and gel filtration and solvent or salt precipitation. The intermediate material

Where the linking agent of formula (II) contains an amidino phenyl ester group X the agent is preferably first reacted with a polypeptide enzyme via the group X and the reaction is preferably carried out under the conditions described for the introduction of blocking groups in European Published Patent Application No. 0,009,879. Having been freed of excess reagent by suitable techniques such as high performance size exclusion chromatography or diafiltration, the acylated enzyme may then be reacted with the other polypeptide at a ratio of approximately 1:1 in a non-nucleophilic buffer at pH7.0-8.0 and $0^{o}$-$30^{o}$C for up to 6h. However, it is preferable to conduct the coupling below $10^{o}$C (preferably $0^{o}$-$4^{o}$C) in order to minimise the hydrolysis of the acylated enzyme.

Where a protein attachment group is introduced in this way, the bridging group (I) will be formed from a reaction of the linking agent (II) and the protein attachment group.

The polypeptides to be linked are reacted separately with the linking agent or the reagent for introducing a protein attachment group by typically adding an excess of the reagent to the polypeptide, usually in a neutral or moderately alkaline buffer, and after reaction removing low molecular weight materials by gel filtration or dialysis. The precise conditions of pH, temperature, buffer and reaction time will depend on the nature of the reagent used and the polypeptide to be modified. The polypeptide linkage reaction is preferably carried out by mixing the modified polypeptides in neutral buffer in an equimolar ratio. Other reaction conditions e.g. time and temperature, should be chosen to obtain the desired degree of linkage. If thiol exchange reactions are involved, the reaction should preferably be carried out under an atmosphere of nitrogen. Preferably, UV-active products are produced (eg from the release of pyridine 2-thione from 2-pyridyl dithio derivatives) so that coupling can be monitored.

After the linkage reaction, the polypeptide conjugate can be isolated by a number of chromatographic procedures such as gel filtration, ion-exchange chromatography, affinity chromatography or hydrophobic interaction chromatography. These procedures my be either low pressure or high performance variants.

The conjugate may be characterised by a number of techniques including low pressure or high performance gel filtration, SDS polyacrylamide gel electrophoresis or isoelectric focussing.

Membrane binding elements which are fatty acid derivatives are attached post translationally to a peptidic membrane binding element, preferably at the terminus of the polypetide chain. Preferably, where the recombinant polypeptide portion of the derivative of the invention contains the peptidic membrane binding element, it has a unique cysteine for coupling to the fatty acid derivative. Where the recombinant

refolded recombinant protein at a late stage in purification (but not necessarily the final stage) and at a reagent concentration preferably below the critical micelle concentration. One of the fatty acid derivative and the recombinant peptide will have the thiol group activated as described above for thiol interchange reactions. The fatty acid derivative is preferably a $C_{10-20}$ fatty acyl derivative of an amino$C_{2-6}$alkane thiol (optionally C-substituted) such as N-(2-myristoyl ) aminoethanethiol or N-myristoyl L-cysteine and forms part of the invention.

Suitable examples of hydrophilic synthetic polymers include polyethyleneglycol (PEG), preferably α,ω functionalised derivatives, more preferably α-amino , ω-carboxy-PEG of molecular weight between 400 and 5000 daltons which are linked to the polypeptide for example by solid-phase synthesis methods (amino group derivatisation) or by thiol-interchange chemistry.

Membrane binding elements derived from ligands of known integral membrane proteins, either amino acid sequences or carbohydrates, may be generated by post-translational modification using the glycosylation pathways of eukaryotic cells targeted to N-linked glycosylation sites in the peptide sequence.

Convenient generic final stage purification strategies are hydrophobic interaction chromatography (HIC ) on C2-C8 media and cation exchange chromatography for separation of derivatised and underivatised proteins into which a hydrophobic-electrostatic switch combination has been inserted.

The derivatives of this invention are preferably administered as pharmaceutical compositions.

Accordingly, the present invention also provides a pharmaceutical composition comprising a derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures for administration by any route, such as oral, topical, parenteral, sublingual or transdermal or by inhalation. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral or sterile parenteral solutions or suspensions or in the form of a spray, aerosol or other conventional method for inhalation.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as

present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrollidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. Tablets may also contain agents for the stablisation of polypeptide drugs against proteolysis and absorbtion-enhancing agents for macromolecules. The tablets may be coated according to methods well known in normal pharmaceutical practice.

Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, is dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Parenteral formulations may include sustained-release systems such as encapsulation within microspheres of biodegradable polymers such as poly-lactic co-glycolic acid.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

Compositions of this invention may also suitably be presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns for example diameters in the range of 1-50 microns, 1-10 microns or 1-5 microns. Where appropriate, small amounts of anti-asthmatics and bronchodilators, for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives

such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included.

Microfine powder formulations may suitably be administered in an aerosol as a metered dose or by means of a suitable breath-activated device.

Suitable metered dose aerosol formulations comprise conventional propellants, cosolvents, such as ethanol, surfactants such as oleyl alcohol, lubricants such as oleyl alcohol, desiccants such as calcium sulphate and density modifiers such as sodium chloride.

Suitable solutions for a nebulizer are isotonic sterilised solutions, optionally buffered, at for example between pH 4-7, containing up to 20mg $ml^{-1}$ of compound but more generally 0.1 to 10mg $ml^{-1}$, for use with standard nebulisation equipment.

The quantity of material administered will depend upon the potency of the derivative and the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, an effective amount of the polypeptide for the treatment of a disease or disorder is in the dose range of 0.01-100mg/kg per day, preferably 0.1mg-10mg/kg per day, administered in up to five doses or by infusion.

No adverse toxicological effects are indicated with the compounds of the invention within the above described dosage range.

The invention also provides a derivative of the invention for use as a medicament.

The invention further provides a method of treatment of disorders amenable to treatment by a soluble peptide which comprises administering a soluble derivative of said soluble peptide according to the invention, and the use of a derivative of the invention for the preparation of a medicament for treatment of such disorders.

In one preferred aspect the present invention relates to derivatives for use in the therapy of disorders involving complement activity and various inflammatory and immune disorders.

In this preferred aspect the soluble polypeptide which is derivatised in accordance with the invention is a soluble complement inhibitor such as a soluble CR1 polypeptide fragment.

Constituting about 10% of the globulins in normal serum, the complement system is composed of many different proteins that are important in the immune system's response to foreign antigens. The complement system becomes activated when its primary components are cleaved and the products alone or with other proteins, activate additional complement proteins resulting in a proteolytic cascade. Activation of the complement system leads to a variety of responses including increased vascular

opsonization of foreign particles, direct killing of cells and tissue damage. Activation of the complement system may be triggered by antigen-antibody complexes (the classical pathway) or, for example, by lipopolysaccharides present in cell walls of pathogenic bacteria (the alternative pathway).

Complement receptor type 1 (CR1) has been shown to be present on the membranes of erythrocytes, monocytes/macrophages, granulocytes, B cells, some T cells, splenic follicular dendritic cells, and glomerular podocytes. CR1 binds to the complement components C3b and C4b and has also been referred to as the C3b/C4b receptor. The structural organisation and primary sequence of one allotype of CR1 is known (Klickstein *et al.*, 1987, J. Exp. Med. 165:1095-1112, Klickstein *et al.*, 1988, J. Exp. Med. 168:1699-1717; Hourcade *et al.*, 1988, J. Exp. Med. 168:1255-1270, WO 89/09220, WO 91/05047). It is composed of 30 short consensus repeats (SCRs) that each contain around 60-70 amino acids. In each SCR, around 29 of the average 65 amino acids are conserved. Each SCR has been proposed to form a three dimensional triple loop structure through disulphide linkages with the third and first and the fourth and second half-cystines in disulphide bonds. CR1 is further arranged as 4 long homologous repeats (LHRs) of 7 SCRs each. Following a leader sequence, the CR1 molecule consists of the N-terminal LHR-A, the next two repeats, LHR-B and LHR-C, and the most C-terminal LHR-D followed by 2 additional SCRs, a 25 residue putative transmembrane region and a 43 residue cytoplasmic tail.

Based on the mature CR1 molecule having a predicted N-terminal glutamine residue, hereinafter designated as residue 1, the first four SCR domains of LHR-A are defined herein as consisting of residues 2-58, 63-120, 125-191 and 197-252, respectively, of mature CR1.

Several soluble fragments of CR1 have been generated via recombinant DNA procedures by eliminating the transmembrane region from the DNAs being expressed (WO 89/09220, WO 91/05047). The soluble CR1 fragments were functionally active, bound C3b and/or C4b and demonstrated Factor I cofactor activity depending upon the regions they contained. Such constructs inhibited *in vitro* complement-related functions such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. A particular soluble construct, sCR1/pBSCR1c, also demonstrated *in vivo* activity in a reversed passive Arthus reaction (WO 89/09220, WO 91/05047; Yeh *et al.*, 1991, J. Immunol. 146:250), suppressed post-ischemic myocardial inflammation and necrosis (WO 89/09220, WO 91/05047; Weisman *et al.*, Science, 1990, 249:146-1511; Dupe, R. *et al.* Thrombosis & Haemostasis (1991) 65(6) 695.) and extended survival [illegible] 1991, J. Surg. Res. 50:350; Pruitt *et*

with p-anisoylated human plasminogen-streptokinase-activator complex (APSAC) resulted in similar anti-haemolytic activity as sCR1 alone, indicating that the combination of the complement inhibitor sCR1 with a thrombolytic agent was feasible (WO 91/05047).

The soluble CR1 polypeptide fragment encoded by sCR1/pBSCR1c, residues 1-1929 of CR1, may be derivatised in accordance with the invention.

Soluble polypeptides corresponding to part of CR1 have been found to possess functional complement inhibitory, including anti-haemolytic, activity. WO94/00571 discloses soluble polypeptides comprising, in sequence, one to four short consensus repeats (SCR) selected from SCR 1, 2, 3 and 4 of long homologous repeat A (LHR-A) as the only structurally and functionally intact SCR domains of CR1 and including at least SCR3.

In preferred aspects, the polypeptide comprises, in sequence, SCR 1, 2, 3 and 4 of LHR-A or SCR 1, 2 and 3 of LHR-A as the only structurally and functionally intact SCR domains of CR1.

In one aspect, the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^1\text{-SCR1-}W^1\text{-SCR2-}X^1\text{-SCR3-}Y^1\text{-OH} \qquad (A)$$

in which SCR1 represents residues 2-58 of mature CR1, SCR2 represents residues 63-120 of mature CR1, SCR3 represents residues 125-191 of mature CR1, and $V^1$, $W^1$, $X^1$ and $Y^1$ represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (I), $W^1$, $X^1$ and $Y^1$ represent residues 59-62, 121-124 and 192-196, respectively, of mature CR1 and $V^1$ represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In another aspect the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^2\text{-SCR1-}W^2\text{-SCR2-}X^2\text{-SCR3-}Y^2\text{-SCR4-}Z^2\text{OH} \qquad (B)$$

in which SCR1, SCR2 and SCR3 are as hereinbefore defined, SCR4 represents residues 197-252 of mature CR1 and $V^2$, $W^2$, $X^2$, $Y^2$ and $Z^2$ represents bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In preferred embodiments of formula (II), $W^2$, $X^2$, $Y^2$ and $Z^2$ represent residues 59-62, 121-124, 192-196, and residues 253 respectively, of mature CR1 and $V^2$ represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In one particular embodiment of formula (B) arginine 235 is replaced by

In one further aspect, the polypeptide may be represented symbolically as follows:

$NH_2$-$X^3$-SCR3-$Y^3$-OH (C)

in which SCR3 is as hereinbefore defined and $X^3$ and $Y^3$ represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (C) $X^3$ represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and $Y^4$ represents amino acids 192-196 of mature CR1.

In another further aspect, the polypeptide may be represented symbolically as follows:

$NH_2$-$X^4$-SCR3-$Y^4$-SCR4-$Z^4$-OH (D)

in which SCR3 and SCR4 are as hereinbefore defined and $X^4$, $Y^4$ and $Z^4$ represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (D) $X^4$ represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and $Y^4$ and $Z^4$ represent amino acids 192-196 and 253 respectively of mature CR1.

The soluble CR1 polypeptide is derivatised in accordance with the invention by any convenient strategy from those outlined above. In a preferred embodiment the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from:

DGPKKKKKKSPSKSSGC

GSSKSPSKKKKKKPGDC

CDGPKKKKKKSPSKSSK

SKDGKKKKKKSKTKC

CSAAPSSGFRILLLKV

AAPSVIGFRILLLKVAGC

and

DGPSEILRGDFSSC

(N-terminus on left).

The soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention is useful in the treatment of many complement-mediated or complement-related diseases and disorders including, but not limited to, those listed

**Disease and Disorders Involving Complement**

**Neurological Disorders**

multiple sclerosis
stroke
Guillain Barré Syndrome
traumatic brain injury
Parkinson's disease
allergic encephalitis
Alzheimer's disease

**Disorders of Inappropriate or Undesirable Complement Activation**

haemodialysis complications
hyperacute allograft rejection
xenograft rejection
corneal graft rejection
interleukin-2 induced toxicity during IL-2 therapy
paroxysmal nocturnal haemoglobinuria

**Inflammatory Disorders**

inflammation of autoimmune diseases
Crohn's Disease
adult respiratory distress syndrome
thermal injury including burns or frostbite
uveitis
psoriasis
asthma
acute pancreatitis

**Post-Ischemic Reperfusion Conditions**

myocardial infarction
balloon angioplasty
atherosclerosis (cholesterol-induced) & restenosis
hypertension
post-pump syndrome in cardiopulmonary bypass or renal haemodialysis
renal ischemia

**Infectious Diseases or Sepsis**
multiple organ failure
septic shock

**Immune Complex Disorders and Autoimmune Diseases**
rheumatoid arthritis
systemic lupus erythematosus (SLE)
SLE nephritis
proliferative nephritis
glomerulonephritis
haemolytic anemia
myasthenia gravis

**Reproductive Disorders**
antibody- or complement-mediated infertility

**Wound Healing**

The present invention is also directed to a pharmaceutical composition for treating a disease or disorder associated with inflammation or inappropriate complement activation comprising a therapeutically effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention, and a pharmaceutically acceptable carrier or excipient.

The present invention also provides a method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention.

In the above methods, the subject is preferably a human.

Further provided is the use of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

In order to inhibit complement activation and, at the same time, provide thrombolytic therapy, the present invention provides compositions which further

Preferred thrombolytic agents include, but are not limited to, streptokinase, human tissue type plasminogen activator and urokinase molecules and derivatives, fragments or conjugates thereof. The thrombolytic agents may comprise one or more chains that may be fused or reversibly linked to other agents to form hybrid molecules (EP-A-0297882 and EP 155387), such as, for example, urokinase linked to plasmin (EP-A-0152736), a fibrinolytic enzyme linked to a water-soluble polymer (EP-A-0183503). The thrombolytic agents may also comprise muteins of plasminogen activators (EP-A-0207589). In a preferred embodiment, the thrombolytic agent may comprise a reversibly blocked *in vitro* fibrinolytic enzyme as described in U.S. Patent No. 4,285,932. A most preferred enzyme is the p-anisoyl plasminogen-streptokinase activator complex, anistreplase as described in U.S. Patent No. 4,808,405 (Monk *et al.*, 1987, Drugs 34:25-49).

Routes of administration for the individual or combined therapeutic compositions of the present invention include standard routes, such as, for example, intravenous infusion or bolus injection. Active complement inhibitors and thrombolytic agents may be administered together or sequentially, in any order.

The present invention also provides a method for treating a thrombotic condition, in particular acute myocardial infarction, in a human or non-human animal. This method comprises administering to a human or animal in need of this treatment an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention and an effective amount of a thrombolytic agent.

Also provided is the use of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention and a thrombolytic agent in the manufacture of a medicament for the treatment of a thrombotic condition in a human or animal. Such methods and uses may be carried out as described in WO 91/05047.

This invention further provides a method for treating adult respiratory distress syndrome (ARDS) in a human or non-human animal. This method comprises administering to the patient an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention.

The invention also provides a method of delaying hyperacute allograft or hyperacute xenograft rejection in a human or non-human animal which receives a transplant by administering an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention. Such administration may be to the patient or by application to the transplant prior to implantation.

routes, an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide derivative according to this invention.

In another preferred aspect the soluble polypeptide is a thrombolytic agent such as prourokinase, streptokinase, tissue-type plasminogen activator or reteplase and the derivative of the invention is useful in the treatment of thrombotic disorders such as acute myocardial infarction. The invention thus provides a pharmaceutical composition for treating thrombotic disorders comprising a therapeutically effective amount of a derivative of a thrombolytic agent according to the invention, and a pharmaceutically acceptable carrier or excipient. The invention further provides a method of treatment of thrombotic disorders by administering an effective amount of a derivative of a thrombolytic agent according to the invention, and the use of such derivative in the preparation of a medicament for the treatment of thrombotic disorders.

The following Methods and Examples illustrate the invention.

## GENERAL METHODS USED IN EXAMPLES

### (i) DNA Cleavage

Cleavage of DNA by restriction endonucleases was carried out according to the manufacturer's instructions using supplied buffers. Double digests were carried out simultaneously if the buffer conditions were suitable for both enzymes. Otherwise double digests were carried out sequentially where the enzyme requiring the lowest salt condition was added first to the digest. Once the digest was complete the salt concentration was altered and the second enzyme added.

### (ii) DNA ligation

Ligations were carried out using T4 DNA ligase purchased from Promega, as described in Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour Laboratory Press.

### (iii) Plasmid isolation

Plasmid isolation was carried out by the alkaline lysis method described in Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour Laboratory Press or by one of two commercially available kits: the Promega Wizard™ *Plus* Minipreps or Qiagen Plasmid Maxi kit according to the manufacturer's instructions.

### (iv) DNA fragment isolation

DNA fragments were excised from agarose gels and DNA extracted using one of three commercially available kits: the QIAEX gel extraction kit or Qiaquick gel extraction kit (QIAGEN Inc., USA), or GeneClean (Bio 101 Inc, USA) according to the manufacturer's instructions.

### (v) Introduction of DNA into *E. coli*

Plasmids were transformed into *E. coli* BL21(DE3) (Studier and Moffat, (1986), *J. Mol. Biol* 189:113), *E. coli* XLI-blue, BL21 (DE3) pLys-S or BLR (DE3) pLys-S that had been made competent using calcium chloride as described in Sambrook *et al*, (1989). Cell lines were purchased as frozen competent cultures from Stratagene. *E. coli* JM109 was purchased as a frozen competent culture from Promega.

**(vi) DNA sequencing**

Plasmid DNA was sequenced on a Vistra DNA Labstation 625. The sequencing chemistry was performed using Amersham International's 'Thermo Sequenase fluorescent dye-terminator cycle sequencing kit' (RPN 2435), in conjunction with their 'FMP fluorescent dye-terminator precipitation kit' (RPN 2433) according to the manufacturer's instructions.

The sequences produced by the above procedure were analysed by a Perkin Elmer ABI Prism 377 DNA Sequencer. This is an electrophoretic technique using 36 cm x 0.2mm 4% acrylamide gels, the fluorescently labeled DNA fragments being detected by a charge coupled device camera according to the manufacturer's instructions.

**(vii) Production of oligonucleotides**

Oligonucleotides were purchased from Cruachem.

**(viii) pBROC413**

The plasmid pT7-7 [Tabor, S (1990), Current Protocols in Molecular Biology, F. A. Ausubel, Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl,eds.] pp.16.2.1-16.2.11, Greene Publishing and Wiley-Interscience,New York.] contains DNA corresponding to nucleotides 2065-4362 of pBR322 and like pBR322 can be mobilized by a conjugative plasmid in the presence of a third plasmid ColK. A mobility protein encoded by ColK acts on the *nic* site at nucleotide 2254 of pBR322 initiating mobilization from this point. pT7-7 was digested with *Lsp*I and *Bgl*II and the protruding 5' ends filled in with the Klenow fragment of DNA PolymeraseI. The plasmid DNA fragment was purified by agarose gel electrophoresis, the blunt ends ligated together and transformed into *E. coli* DH1 by electroporation using a Bio-Rad Gene Pulser and following the manufacturers recommended conditions. The resultant plasmid pBROC413 was identified by restriction enzyme analysis of plasmid DNA.

The deletion in pBROC413 from the *Lsp*I site immediately upstream of the f 10 promoter to the *Bgl*II site at nucleotide 434 of pT7-7 deletes the DNA corresponding to nucleotides 2065-2297 of pBR322. The *nic* site and adjacent sequences are therefore deleted making pBROC413 non mobilizable.

**(ix) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)**

SDS PAGE was carried out generally using the Novex system (British Biotechnology) according to the manufacturer's instructions. Prepacked gels of 4 - 20% [illegible] including protein molecular weight

1%(w/v)SDS - containing buffer (with or without 5%(v/v) 2-mercaptoethanol), and left at room temperature for about 10 to 30 min before application to the gel.

**(x) Reduction of disulphides and modification of thiols in proteins**

There are a number of methods used for achieving the title goals. The reason it may be necessary to carry out selective reduction of disulphides is that during the isolation and purification of multi-thiol proteins, in particular during refolding of fully denatured multi-thiol proteins, inappropriate disulphide pairing can occur. In addition, even if correct disulphide paring does occur, it is possible that a free cysteine in the protein may become blocked, for example with glutathione. These derivatives are generally quite stable. In order to make them more reactive, for example for subsequent conjugation to another functional group, they need to be selectively reduced, with for example dithiothreitol (DTT) or Tris (2-carboxyethyl) phosphine.HCl (TCEP) then optionally modified with a function which is moderately unstable. An example of the latter is Ellmans reagent (DTNB) which gives a mixed disulphide. In the case where treatment with DTNB is omitted, careful attention to experimental design is necessary to ensure that dimerisation of the free thiol-containing protein is minimised. Reference to the term 'selectively reduced' above means that reaction conditions eg. duration, temperature, molar ratios of reactants have to be carefully controlled so that reduction of disulphide bridges within the natural architecture of the protein is minimised. All the reagents are commercially available eg. from Sigma or Pierce.

The following general examples illustrate the type of conditions that may be used and that are useful for the generation of free thiols and their optional modification. The specific reaction conditions to achieve optimal thiol reduction and/or modification are ideally determined for each protein batch.

TCEP may be prepared as a 20mM solution in 50mM Hepes (approx. pH 4.5) and may be stored at -40 degrees C. DTT may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. DTNB may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. All of the above reagents are typically used at molar equivalence or molar excess over protein concentration, the precise concentrations ideally identified experimentally. The duration and the temperature of the reaction are similarly determined experimentally. Generally the duration would be in the range 1 to 24 hours and the temperature would be in the range 2 to 30 degrees C. Excess reagent may be conveniently removed by buffer exchange, for example using Sephadex G25 or Sephadex G50. A suitable buffer is 0.1M sodium

## EXAMPLES

**Example 1 Preparation of N-(Myristoyl) 2-aminoethane thiol (MAET)**

Myristoyl chloride (1.0 mmol)was added with vigorous stirring to ice-cooled dry pyridine (1.0ml), and followed immediately by N-hydroxysuccinimide (1.5 mmol).The mixture was stirred for 4h at ambient termperature (~23°C). 2-aminoethanethiol free base (1.1 mmol) was added as solid to the mixture and allowed to react for 6h at ambient temperature, followed by 3 days at 4°C. The product was treated with water (5ml), stirred for 1h at ambient and filtered, washing with cold water. The white solid was dissolved in dimethylsulphoxide and reprecipitated with water and then vacuum dried over phosphorous pentoxide. The final yield was 0.21g (~70%). Thiol titration using Ellman's reagent indicated that the product contained ~45% free thiol.

**Example 2 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 1 (MSWP-1) (SEQ ID NO: 27)**

**N-(Myristoyl) -Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(S-2-Thiopyridyl)Cys-$NH_2$**

The peptide:

Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$

(SEQ ID NO: 5)

was prepared using solid phase synthesis via the general Fmoc/tBu strategy developed by Sheppard and Atherton (E.Atherton and R.C.Sheppard, Solid Phase Synthesis, IRL Press, Oxford, 1989). Kieselguhr-supported polydimethylacrylamide resin (Macrosorb 100) was used as the solid support and was derivatised with ethylene diamine.

Coupling reactions were carried out using N-α-Fmoc protected reagents pre-activated with N,N'-diisopropylcarbodiimide/ N-hydroxybenzotriazole (in 4-fold molar excess) with bromophenol blue monitoring. Fmoc cleavages used 20% piperidine in DMF. Reactions to assemble the peptide chain were carried out by repeated cycles of coupling and deprotection including the attachment of the modified Rink linkage reagent (p-[(R,S)-α-[1-(9H-fluoreny-9-yl-methoxyformamido ] 2,4 dimethoxybenzyl]-phenoxyacetic acid) designed to yield a C-terminal amide on final cleavage. The side chain functionalities of the individual amino-acids were protected as follows:

Ser (tButyl), Lys (Boc), Asp (O-tButyl), Cys (Trityl).

On completion of the peptide assembly and with the peptide still attached to the resin, the myristoyl group was attached to the amino group of the N terminal glycine by [illegible] This modified peptide

same time by treatment with trifluoracetic acid containing 2.5% water and 2.5% triisopropyl silane.

The crude product was treated with 2,2' dithiopyridine in 0.01M ammonium acetate solution at pH 8-9 for approx. 2h, then acidified with acetic acid and purified by preparative high performance liquid chromatography (HPLC) in 0.1% trifluoracetic acid (TFA) /water and 0.1% TFA/acetonitrile as gradient component. After lyophilisation, the peptide was a white amorphous powder, soluble to at least 10mg/ml in dimethylsulphoxide. Fast atom bombardment mass spectrometry gave main peaks at m/e 2107.8, 2129.7 and 2145.8, corresponding to the monoprotonated, monosodiated and monopotassiated molecular ions of the peptide. The 2-thiopyridyl content of the peptide was measured by dissolving it to around 0.03mM to 0.2 mM in 0.1M Sodium Borate pH 8.0 and reducing by addition of dithiothreitol to 5mM. The change in optical density at 343nm was used to calculate the amount of pyridine 2-thione released using an extinction coefficient at this wavelength of 8080 $cm^{-1}$ $M^{-1}$ . This indicated that the peptide content was approximately 60% of the dry weight.

**Example 3 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 2 (MSWP-2) (SEQ ID NO: 28)**

**N-acetyl-Cys (2-thiopyridyl) Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser Lys-Ser-Ser-Lys-(ε-N-(Myristoyl))-$NH_2$**

The peptide:
Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Lys-$NH_2$
(SEQ ID NO: 18) was prepared by solid-phase synthesis using the general method described in Example 2 and with the following variations:

a. The C-terminal lysine was protected by alkylation with the 4-methyl trityl (MTT) group; all other lysines were N-ε protected with the t-Boc group

b. MTT was removed with 1% v/v trifluoracetic acid in dichloromethane and the resulting unique free amino group derivatised with myristic acid prior to deprotection of the other lysines (as described in Example 2)

The N-terminus was acetylated with acetic anhydride upon completion of the peptide chain assembly. Generation of the 2-pyridyldithiocysteine moiety was by reation of the deprotected peptide with 2,2'-dithiopyridine as described above. The product was purified as described in Example 2. Fast-atom bombardment mass spectrometry gave a molecular ion peak at 2221.3 (cf 2220.3 for the monoprotonated theoretical mass).

Amino-acid Analysis:

| | Asx | Ser | Gly | Pro |
|---|---|---|---|---|
| Theory: | 1.0 | 4.0 | 1.0 | 2.0 |
| Found | 0.97 | 3.53 | 1.15 | 1.88 |

(Asx = Asn or Asp)

Amino-acid analysis indicated a net peptide content by weight of 68.7%. The 2-pyridyl disulphide content was appoximately 60% by weight using the method of Example 2.

**Example 4 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 3 (MSWP-3) (SEQ ID NO: 29)**

**N-(Myristoyl) -Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-(S-2-Thiopyridyl)Cys-$NH_2$**

The peptide:

Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser -Lys-Thr-Lys-Cys (SEQ ID NO: 19) was prepared using the general solid-phase synthesis protocol of Example 2. Myristoylation, C-terminal amidation and derivatisation of the Cys residue were performed as described in Example 2. After purification, mass spectrometry gave the major peak at 2040.5, corresponding to a monoprotonated form (Theory: 2039.5)

Amino-acid analysis:

| | Asx | Ser | Gly | Thr | Lys |
|---|---|---|---|---|---|
| Theory: | 1 | 2 | 1 | 1 | 9 |
| Found: | 1.02 | 2.04 | 1.14 | 1.06 | 8.85 |

The peptide content was about 56% by weight

**Example 5 Synthesis of T-Cell Targeting Peptide Reagent 1 (TCTP-1) (SEQ ID NO: 30)**

**N-acetyl-(2-thiopyridyl)Cys Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val-$CONH(CH_2)_9CH_3$**

The peptide Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val (SEQ ID NO: 20) was prepared using the general solid-phase methodology of Example 2 and N-acetylated as in Example 3. The C-terminus was derivatised using n-decylamine in place of the Rink reagent. Mass spectrometry of the purified peptide gave

1951.1.) An ion at 1843.3 was also observed , this is believed to correspond to loss of the thiopyridyl group in the spectrophotometer.

Amino-acid analysis:

| | Ser | Gly | Arg | Ala | Pro | Val | Ile | Phe | Leu | Lys |
|---|---|---|---|---|---|---|---|---|---|---|
| Theory: | 3 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 3 | 1 |
| Found: | 2.95 | 1.10 | 1.10 | 2.11 | 1.04 | 0.60 | 0.92 | 1.00 | 3.03 | 1.03 |

The peptide content by weight was 53%

**Example 6 Expression and isolation of [SCR1-3]-Cys (SEQ ID NO: 6)**

**(a) Construction of plasmid pDB1030 encoding [SCR 1-3]-Cys**

The plasmid coding for SCR1-3 of LHR-A of CR1, pDB1013-5 (patent application WO 94/00571) was digested with restriction endonucleases *Eco*RI and *Hin*dIII and the 2.2 kB plasmid band was isolated from an agarose gel using a Qiagen Qiaex DNA extraction kit according to the manufacturer's instructions. This is fragment 1. A second batch of pDB1013-5 was digested with *Ban*I and *Eco*RI and the 196 bp band was extracted from agarose as above. This is fragment 2. Two oligonucleotides, SEQ ID No.1 and SEQ ID No.2, were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has a *BanI*/*Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains a codon coding for cysteine just before the stop codon. This is fragment 3.

Fragments 1,2 and 3 were ligated with T4 DNA ligase in a single reaction to give pDB1030 . The ligated plasmid was transformed into competent *E. coli* JM109 purchased from Promega. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR(1-3) (SEQ ID No.27 of WO 94/00571) had been altered by a single C-terminal cysteine residue to give SEQ ID No.6.

**(b) Expression of [SCR1-3]-Cys from pDB1030**

pDB1030 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1030 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L $Na_2HPO_4$, 0.1g/L $KH_2PO_4$) containing 50ug/ml ampicillin. The culture was grown for 6 hours at [illegible] to inoculate 100 ml of the same media containing 50

culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at $A_{600}$ nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. Pellet from 2L of culture was stored at -80°C

**(c) Isolation, refolding, purification and formulation of [SCR1-3]-Cys**

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736.

**i) Isolation of solubilised inclusion bodies**

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1030) was resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker surrounded by ice and sonicated (Heat systems - Ultrasonics W380; 50 x 50% pulse, pulse time = 5 sec.) for typically 3 - 6 minutes. The disrupted pellet was then frozen and stored at -80°C. Approx. 2 weeks later the sonicate was thawed and centrifuged at approx. 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (200ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by overnight at 4° C.

**ii) Initial purification using SP-Sepharose**

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction-dried. The mixture was swirled vigorously and left static for 1-2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0.05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the $A_{280}$ of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single $A_{280}$ peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein concentration of the solution was estimated by $A_{280}$ determination, using a molar extinction coefficient of 25000 $cm^{-1}$ of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 1.6mg/ml. The solution was stored at -40°C.

**iii) Folding and further processing**

25ml of the SP-Sepharose-purified product was added gradually over a 1 min [illegible] 0.02M ethanolamine/1mM EDTA with

to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 35 ml; the retentate was slightly cloudy and had the appearance of a translucent solution. It was stored for 12 days at 4° C. It was then spun at 30 000g for 15 mins and the supernatant mixed with 9 vol. 0.1M $NaH_2PO_4$/1M $(NH_4)_2S0_4$ pH 7.0 (Buffer A) at room temperature and immediately centrifuged at 3000 rpm for 15 min. The supernatant was ultrafiltered (YM10) to about 4ml and then buffer-exchanged into 0.1M sodium phosphate pH 7.0 (5.0ml); this solution had a protein concentration of 1.7mg/ml by A280 analysis. It was treated with dithio bis nitrobenzoic acid (DTNB) (8-fold molar excess) for 30 min at room temperature. Free thiol content based on A412 measurement and an extinction coefficient (for the free thionitrobenzoate ion) of 13 600 was 6uM equivalent to only about 10% derivatisation to give Product A. The majority of the product was believed to be [SCR1-3]-Cys where the free C-terminal thiol was blocked by reaction with glutathione or 2-mercaptoethanol during the refolding stage.

**(d) Alternative method for isolation, refolding, purification and formulation of [SCR1-3]-Cys**

The method was similar to that described above, except that it more closely followed the procedures described in Dodd et al (op cit.). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Toyopearl chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as Product B. Starting with a nominal 100mg of fully reduced SCR1-3/cys, Product B contained 17mg. The product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular weight of 21 000. On the basis of studies with similarly produced preparations it was believed to be the S-glutathione and/or S-mercaptoethanol derivatised form of the parent protein, although at least some batches produced in a similar way or stored for a period of time might exist as the free cysteine variant. The product also contained a polypeptide with an apparent molecular weight of about 40 000 On the basis of studies with similar batches of protein enriched in this species it was identified as the dimer of [SCR1-3]-Cys.

**Example 7 Expression and isolation of SCR1-3/switch fusion (SEQ ID NO: 7)**

**(a) Construction of plasmid pDB1031 encoding SCR1-3/switch**

Fragment 1 and fragment 2 of pDB1013-5 were the same as Example 6 above. Two oligonucleotides, SEQ ID No.3 and SEQ ID No. 4, prepared by Cruachem were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has an *BanI/Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains 17 additional codons coding for DGPKKKKKKSPSKSSGC just before the stop codon. This is fragment 4.

Fragments 1, 2 and 4 were ligated with T4 DNA ligase in a single reaction to give pDB1031. The ligated plasmid was transfomed into competent *E. coli* JM109. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR1-3 (SEQ ID 27 of WO 94/00571) had been altered by C terminal addition of amino acids DGPKKKKKKSPSKSSGC to give SEQ ID NO: 7.

**(b) Expression of SCR1-3/switch from pDB1031**

pDB1031 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1031 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L $Na_2HPO_4$, 0.1g/L $KH_2PO_4$) containing 50ug/ml ampicillin. The culture was grown for 6 hours at 37°C, 230 r.p.m. before being used to inoculate 100 ml of the same media containing 50 ug/ml ampicillin. Growth was under the same conditions overnight. 25 ml of each culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at $A_{600}$ nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. The cell pellet was frozen at -40 degrees C.

**(c) Isolation, refolding, purification and formulation of SCR1-3/switch**

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736, with some modifications.

**i) Isolation of solubilised inclusion bodies**

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1031) was thawed and resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker [illegible] Ultrasonics W380: 50 x 50% pulse

stored at -80°C. Approx. 1d later the sonicate was thawed and centrifuged at approx. 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (240ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by 5 days at 4° C.

ii) **Preliminary purification using SP-Sepharose**

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction dried. The mixture was swirled vigorously and left static for approx. 2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0.05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the $A_{280}$ of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single $A_{280}$ peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein concentration of the solution was estimated by $A_{280}$ determination, using a molar extinction coefficient of 25000 $cm^{-1}$,of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 2.8mg/ml. Analysis by SDS PAGE/stain showed a major band (approx 80%) at about 23 000Da. The solution was stored at -40°C.

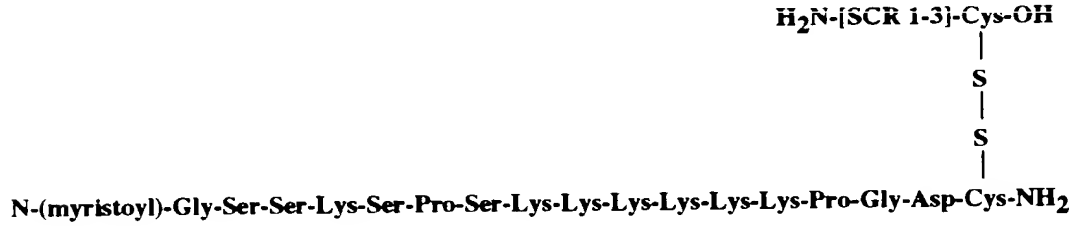
iii) **Folding and further processing**

14ml of the SP-Sepharose-purified product was added gradually over a 1 min period to 430 ml freshly prepared, cold 0.05M Hepes/2 M sodium chloride/1mM EDTA pH 8.0 with continuous swirling, and left static for 1 h/4°C. Reduced glutathione (GSH) was added to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 34 ml; the retentate was slightly cloudy. It was then spun at 25 000g for 15 mins and the supernatant buffer-exchanged into 0.1M sodium phosphate pH 7.0 (46 ml). This fraction contained 2 mg of protein on the basis of an A280 determination. The solution was mixed with DTNB (20mM; 0.65ml) for 20 min at 4 degrees C and then ultrafiltered to 2.4ml. This retentate was buffer-exchanged into 0.1M sodium phosphate pH 7.0 (3.0ml) and stored at -40 degrees C. Absorbance measurements at 412nm on the solution prior to ultrafiltration suggested 25% derivatisation with DTNB.

**(d) Alternative isolation, refolding, purification and formulation of SCR1-3/switch**

The method was similar to that described in (c) above, except that following the ultrafiltration step after refolding it more closely followed the procedures described in Dodd et al (*op cit.*). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Sepharose chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as initial product. Additional treatment with TCEP essentially as above, followed by DTNB yielded a final product at 10uM final protein concentration. The final product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular weight of 23 000 and contained about 2 moles TNB per mole of protein.

**Example 8 Preparation of [SCR1-3]-Cys-S-S-[MSWP-1] (SEQ ID NO: 8)**




**(a)** Product A of Example 6(c) (1.5ml) was treated with dithiothreitol (30 ul of 0.5M in water, final concentration 10mM) for 60 min at 4°C to give the free peptide SEQ ID NO 6. The yellow solution was gel filtered at 4°C on a small column of Sephadex G-25 (PD-10, Pharmacia) into 0.05M Hepes.HCl buffer pH 7.5 (3.0ml). The slightly cloudy solution was mixed with a solution of MSWP-1 (Example 2) (3.8mM dithiopyridyl equivalents, 150 ul) to a final concentration of 0.18mM (~8 molar equivalents). The mixture was held for 2h on ice and then gel filtered as before but using 2 PD10 columns (1.6 ml applied, 3.2ml eluted). The final eluate was not cloudy and was stored frozen at -70°C in aliquots of 0.4ml.

**(b)** [SCR1-3]-Cys protein product B described in Example 6(d) (1.5ml; 31uM protein) was mixed with TCEP (20mM; 0.007ml) and incubated at room temperature for 23 h to give the free protein SEQ ID NO: 6. MSWP-1 (Example 2) (10mM; 0.093ml) was added and the solution incubated for a further 4 h. 0.75ml of the final solution was buffer-exchanged into 50mM formic acid and aliquots left in solution or lyophilised. [illegible] SDS PAGE and had an apparent molecular weight of 23

lyophilisate was easily soluble in 50mM formic acid at an estimated protein concentration of 2 mg/ml.

(c) [SCR1-3]-Cys protein product B described in Example 6(d) (21.6ml; 31uM protein) was mixed with TCEP (20mM; 0.1ml) and incubated at room temperature for 22h to give the free protein SEQ ID NO 6. MSWP-1 (20mM in 0.1M sodium phosphate pH 7.0; 0.67ml) was added and the solution incubated for a further 4 h. All 22ml was buffer-exchanged into 50mM formic acid using Sephadex G50 (Vt 160ml). Three A280 peaks were obtained. The first one, eluting at volume 56 -106ml, was the title compound according to SDS PAGE analysis. The fraction was aliquoted and aliquots stored at - 40 degrees C or lyophilised. Amino acid analysis of the pre-lyophilisation solution indicated a protein concentration of 0.42mg/ml. A280 (1cm path length) was 0.44. C8 reverse phase HPLC and SDS PAGE both indicated a purity of approx 80% The latter technique showed the major band had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000; on reduction the 23 000 band shifted to two bands with molecular weights of approx 21 000 and approx 5 000. The lyophilisate was easily soluble in 50mM formic acid or in PBS 'A' (Dulbecco) at a protein concentration of 6mg/ml.

**(d)** [SCR1-3]-Cys-S-S-[MSWP-1] from (c) was divided into 0.3 ml aliquots and freeze-dried. Individual aliquots were resolubilised in 50mM formic acid (0.3ml or 0.039ml).

**Example 9 Preparation of [SCR1-3/switch fusion]disulphide linked to [MAET] (SEQ ID NO: 31)**

**$H_2N$-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH**
**$CH_3(CH_2)_{12}CONH-(CH_2)_2-S-S$**

Title compound can be synthesised using TNB-activated SCR1-3/switch (SEQ ID NO: 7) prepared as in Example 7(d). The TNB-activated SCR1-3/switch is mixed with a molar excess of MAET (Example 1), which might be typically made up at 2.0mg/ml in DMSO, equivalent to about 3mM free thiol. Typical reaction conditions would be 1 to 4 hours at room temperature or overnight at 4 degrees C using a protein concentration of 1 to 100 uM. The reaction may be monitored by checking the generation of yellow colour, which is caused by the release of free TNB ion. Once the reaction is complete the

**Example 10 Preparation of [SCR1-3/switch fusion] disulphide linked to [MSWP_1] (SEQ ID NO: 9)**

$H_2N$-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH
|
S
|
S
|

N-(Myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$

**Method (a)**

0.02ml of MSWP-1 (Example 2, 10mM in 0.1M sodium phosphate pH 7.0) was mixed with 0.005ml of TCEP (20mM in 50mM Hepes) and left for 10 min at room temperature. The resultant solution was Solution A containing the myristoylated peptide of SEQ ID NO: 5. TNB-activated SCR1-3/switch (SEQ ID NO: 7) prepared in a similar way to that described in Example 7(c) (0.3ml; 15uM in 0.1M sodium phosphate pH 7.0) was mixed with 0.0056ml of Solution A to give a theoretical MSWP-SH molar excess of five-fold over protein. The mixture was left for 4h at room temperature followed by 18h at 4 degrees C. Analysis by SDS PAGE followed by protein staining indicated one major band at apparent $M_r$ 23K, corresponding to unreacted protein, and a minor band at apparent $M_r$ 26K, corresponding to title protein.

**Method (b)**

**TNB-activated** SCR1-3/switch product (SEQ ID NO: 7) (10uM; 0.43ml) prepared in a similar way to that described in Example 7(d) was mixed with TCEP (5mM; 0.0026ml) and incubated for 17h at room temperature to yield the free fusion protein SEQ ID NO: 7. MSWP-1 (10mM; 0.0086ml) was added and incubation was continued for a further 4h. Small particles or crystals were present in the solution, but it was otherwise clear. The particulate solution was buffer-exchanged into 50mM formic acid (1.0ml), aliquoted and frozen. Analysis by SDS PAGE under non-reducing conditions showed a number of bands, which included a species with an apparent molecular weight of 25 000 - the target species.

**Example 11 Preparation of [CR1: 1-1929]-Cys-S-S-[MSWP-1] (SEQ ID NO: 10)**

$H_2N$-[CR1(1-1929)]-Cys-OH
|
S
|
S
|

N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$

Human complement receptor 1 (CR1, CD35) is a known regulator of complement activation which has been produced in a recombinant soluble form containing all of the extracellular SCR domains of a major natural allotype (Fearon et al, WO 89/09220, WO 91/05047). This form (sCR1) has been expressed as an active protein in Chinese Hamster Ovary (CHO) cells. Mutagenesis of the DNA sequence immediately downstream of the codon for Cys-1924 is performed to generate a new C-terminal cysteine residue.

A suitable example of a modified terminus of the cDNA sequence of sCR1 is as follows:

(5909) Bal I (5914)

.....CCT CTG GCC AAA TGT ACC TCT CGT GCA CAT TGC TGA

The codon Asp-1930 in CR1 is replaced by that for a Cysteine (followed by a stop codon to generate a soluble protein) through ligation of a modified oligonucleotide to the unique Bal I restriction endonuclease site at position 5914 (numbering from Fearon et al, 1989,1991).

Expression of this modified cDNA in CHO cells and isolation of the product by standard chromatographic procedures generates a modified sCR1 protein which can be treated as in Example 8(a), (b) or (c) to couple it to MSWP-1 (Example 2) to yield the title compound.

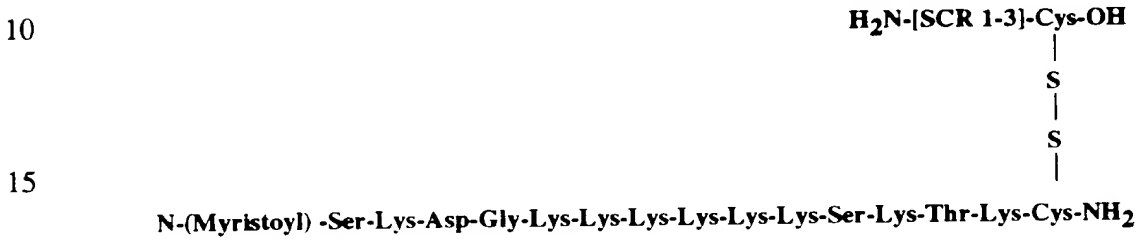
**Example 12 Preparation of [SCR1-3]-Cys-S-S-[MSWP-2] (SEQ ID No. 11)**

$H_2N$-[SCR 1-3]-Cys-OH
|
S
|
S
|

N-acetyl-Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser Lys-Ser-Ser-Lys-(N-Myristoyl)-$NH_2$

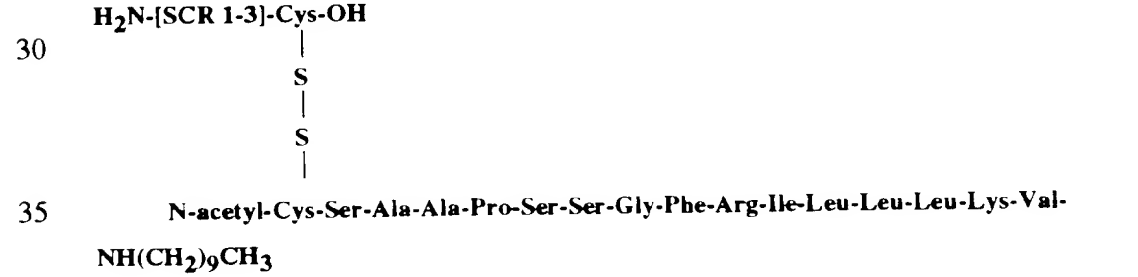
0.0054ml) and incubated at room temperature for approx. 20h. 0.05ml of this solution was mixed with 0.025ml of 0.1M ethanolamine and 0.003ml of MSWP-2 (see Example 3; 5mM in DMSO; ); the solution was incubated for a further 3h at room temperature. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

**Example 13 Preparation of [SCR1-3]-Cys-S-S-[MSWP-3] (SEQ ID No. 12)**




[SCR1-3]-Cys protein (SEQ ID NO: 6) prepared in a similar way to that described in Example 6(d) (46uM protein; 0.10ml) was mixed with TCEP (5mM; 0.0037ml) and incubated at room temperature for approx. 18h. 0.01ml of 0.5M ethanolamine was added. 0.03ml of this 0.11ml solution was mixed with 0.0032ml of MSWP-3 (see Example 4; 2mM in 0.1M sodium phosphate pH 7.0); the solution was incubated for a further 3h at room temperature. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

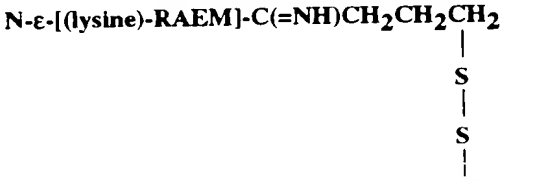
**Example 14 Preparation of [SCR1-3]-Cys-S-S-[TCPT-1] (SEQ ID No. 13)**




[SCR1-3]-Cys protein prepared in a similar way to that described in Example 6(d) (46uM protein; 0.08ml) was mixed with TCEP (5mM; 0.0029ml) and incubated at room

DMSO). The TCPT-1 was added in 6 aliquots over a 2h period to minimise aggregation. The solution was incubated for a further 2h at room temperature. The final appearance of the mixture was one of a colloidal suspension and centrifugation at 2000g for 1 min showed that the target protein was compartmentalised in the precipitate. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of about 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

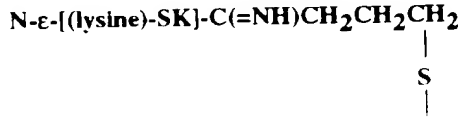
**Example 15 Preparation of a Rabbit anti-(human erythrocyte membrane) antibody - [MSWP-1] conjugate (RAEM-MSWP-1) (SEQ ID NO: 32)**




Rabbit polyclonal anti(human erythrocyte membrane) (RAEM) antiserum (Dako, Denmark, 13mg/ml, 0.25ml) was diluted to 1.0ml with 50mM sodium phosphate 0.1M sodium chloride pH 7.4 (PBS) and treated with 30 ul of 100mM 2-iminothiolane in PBS (freshly dissolved) for 30 min at 25°C. These conditions have been shown (R.A.G.Smith & R.Cassels, Fibrinolysis, **2**,189-195, 1988) to introduce an average of 2-3 free thiol groups per molecule of immunoglobulin G.

The product was purified by gel permeation chromatography on a small disposable column of Sephadex G-25m (PD-10, Pharmacia, Stockholm,Sweden) at 4°C. 2.5ml of the product (total volume 3.0ml, theoretical protein concentration ~6.1 uM) was treated with MSWP-1 (Example 2, 0.125 ml of 5mM solution in dimethyl sulphoxide, final conc ~240uM) and incubated at 25°C for 30 min. The product was gel-filtered on a PD10 column as above to give 3.0ml of a solution ~5uM in protein. This was stored frozen at -70°C.

**Example 16 Preparation of a conjugate of Streptokinase and MSWP-1 (SEQ ID No 21)**



Streptokinase (SK) stock solution (Behringwerke, Marburg, Germany, 12.8 mg/ml, 271 uM, 2.5ml) was gel filtered using a PD10 column into 3.2 ml of PBS buffer (see Example 15) containing 0.01%w/v Tween 80 [PST buffer]. Freshly made up 2-iminothiolane (64ul of 100mM) was added and the mixture incubated at 25°C for 1h. The product was gel filtered in 2 x 1.6ml batches into 2x 3.0 ml PST at 4°C on two PD10 columns. This solution was stored in aliquots of 1.5ml at -75°C.

Titration of the product with Ellman's reagent (0.1mM in 0.5ml 0.1M Triethanolamine.HCl pH 8.0) showed that it contained approximately 0.3mM free thiol groups. This corresponds to an average of 3- 3.5 thiol groups per molecule of SK. The stock thiolated SK solution (2 x 0.5ml) was processed by modifying one aliquot with MSWP-1 (32ul of 5mM stock in DMSO), incubated 1h at 25°C and gel filtered (PD10 column) into 3.0ml PST at 4°C. A control aliquot was processed in parallel without exposure to MSWP-1. Both products contained ~ 0.8 mg/ml protein based on an extinction coefficient of 0.76 $(mg/ml)^{-1}$ at 280nm for SK and were stored at -75°C.

**Example 17 Reversible linkage of MSWP-1 to the active centre of Human Tissue-type Plasminogen Activator (SEQ ID No 22)**

**tPA [Ser-478]-O-4-benzoyl-$NH(CH_2)_2NHCO(CH_2)_2$-S**
|
S
|
**N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$**

The thiol-reactive acyl-enzyme 4-N-[2-N-(3-[2-pyridyldithio]-ethylcarbonyl)aminoethyl]aminobenzoyl - [Ser-478] human tissue-type plasminogen activator [PDAEB->tPA] was prepared by the method of Smith and Cassels (Fibrinolysis, 2, 189-195, 1988). Tissue plasminogen activator (Actilyse, Boehringer Ingelheim,Germany, approx 2mg) was dissolved in the PST buffer of Example 16 (1.0ml) and treated with 25ul of a 20mM solution of 4'-amidinophenyl 4-N-[2-N-(3-[2-pyridyldithio]-ethylcarbonyl)aminoethyl]aminobenzoate hydrochloride (S.B.Kalindjian & R.A.G.Smith, Biochem. J. 248, 409-413,1987) in dimethylsulphoxide. The mixture was incubated for 1h at 25°C and stored frozen at -80°C. It was reduced by addition of dithiothreitol (5ul of 0.5M in water) for 30 min at 0°C followed by buffer-exchange into PST buffer (3.0ml) as described in Example 16. The product was divided immediately into a retained sample (0.6ml) and a reaction sample (2.4ml) which was mixed with MSWP-1 (Example 2, 100ul of a 5mM solution in dimethylsulphoxide) and incubated for 90 min on ice. The product was buffer-exchanged as above into 3.2ml PST and stored

**Example 18 Expression and purification of [SCR1-3]-Cys (SEQ ID 6) from a fermentation run.**

**(a) Fermentation of *E. coli* harbouring the plasmid pDB1030**

A frozen stock of *E. coli* harbouring the plasmid pDB1030 was initially prepared by plating the culture out onto LB agar plus ampicillin at 100µg/ml. 1ml aliquots were preserved in a 10% glycerol / PBS cryopreservative and stored under liquid nitrogen. A 1ml vial was thawed and was used to inoculate 100ml $LB^{Amp100}$ primary seed medium (Difco Bactotryptone, 10$gl^{-1}$; Difco yeast extract, 5$gl^{-1}$; sodium chloride, 5$gl^{-1}$; pH pre-sterilisation 7.4) in a 500ml flask. The primary seed stage was incubated at 37°C for 3 hours before transfer to the second seed stage, also 100ml $LB^{Amp100}$ per 500ml flask using a 1% inoculum. Following incubation as above for a further 4 hours a 1% inoculum was transferred to the tertiary seed stage, 10litres $LB^{Amp100}$ in a 15litre Biolafitte fermenter. The 10 litres tertiary seed medium was sterilised *insitu* for 45 minutes at 121°C before inoculation. Following incubation for 14.5 hours, the tertiary seed was transferred to the final stage fermenter as a 6% inoculum. Incubation conditions for the seed stage were as follows: airflow at 10l$min^{-1}$ (1.0vvm), temperature 37°C, agitation at 400rpm (1.9$ms^{-1}$) and overpressure 0.2bar. 300 litres Tryptone phosphate $medium^{Amp100}$(Difco Bactotryptone, 20$gl^{-1}$; Difco yeast extract, 15$gl^{-1}$; sodium chloride, 8$gl^{-1}$; disodium hydrogen orthophosphate, 2$gl^{-1}$; potassium dihydrogen orthophosphate, 1$gl^{-1}$; Dow Corning 1520 antifoam, 0.1$gl^{-1}$; pH pre-sterilisation 7.4) was sterilised *in situ* for 30 minutes at 121°C in a 450L Bioengineering fermenter. The fermenter was inoculated with 20 litre inoculum from the tertiary seed stage and incubated under the following conditions : airflow 450L $min^{-1}$ (1.5vvm), temperature 37°C, agitation 230rpm (1.5$ms^{-1}$) and overpressure 0.5bar. After an $OD_{550nm}$ of 3.5 was obtained, 1mM IPTG was added. Harvest followed after continued incubation for 2 hours. A cell slurry was recovered after primary centrifugation through a Westfalia CSA19 (two discharges). The cells were further spun at 4700rpm (7000g) for 30 minutes in a Sorvall RC3B centrifuge. The total cell yield (wet weight) was 2.98Kg and was stored at -80 degrees C in approx. 600g lots.

**(b) Isolation of inclusion bodies and purification of [SCR1-3]-Cys**

Inclusion bodies from 100 g (wet weight) cell pellet were isolated and solubilised essentially as described in Example 6. The purification of target protein from resolubilised inclusion bodies was also as described in Example 6 with some modifications. The major ones were :

pure target protein was produced in the solubilised and partially purified fractionon bodies.

2. Refolding of a 100mg sample of the partially purified protein was carried out by diluting the fully denatured protein (2mg/ml) 100-fold in cold 60mM ethanolamine/1mM EDTA, followed by addition of the glutathione redox couple.

The product of the above process was capable of being modified with MSWP-1 (Example 2) in a way similar to that described in Example 8.

**Example 19 Expression and isolation of [SCR1-3(delN195-K196)]TNANKSLSSISCQT (SEQ ID NO: 14)**

**(a) Construction of plasmid pBC04-29 encoding [SCR1-3(delN195-K196)]TNANKSLSSISCQT**

Plasmid pBC04-29 was constructed from plasmid pDB1013-5 encoding SCR1-3 of LHR-A of CR1 (patent application WO 94/00571) by QuickChange site directed mutagenesis (Stratagene) according to the manufacturers protocols. Two complementary oligonucleotides ( SEQ ID No 15 and SEQ ID No 16 were used to generate a novel restriction site (silent) at G186/P187 and a C terminal cysteine. In the event the reaction produced a frame-shift mutation at position N195. In the resulting sequence the C terminal amino acids N195 and K196 are replaced with a 14 amino acid peptide TNANKSLSSISCQT. Fortuitously, this sequence contains an internal cysteine close to the C terminus, preceeded by a spacer sequence of 11 amino acids.

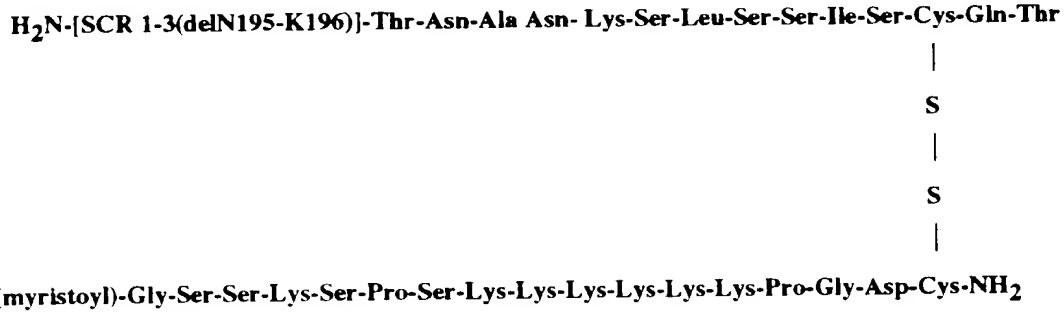
**(b) Expression of plasmid pBC04-29 encoding [SCR1-3(delN195-K196)]TNANKSLSSISCQT in *E. coli***

pBC04-29 was transformed into competent *E. coli* BL21(DE3)pLys-S and resultant colonies were isolated and checked for plasmid content. A single colony was inoculated into 10 ml LB medium (10g/L bactotryptone, 5g/L yeast extract, 10g/L NaCl) containing 50ug/ml ampicillin. The culture was grown for 6 -18 hours at 37$^{o}$C, 230 r.p.m. before being used to inoculate 1 litre of the same medium containing 50 ug/ml ampicillin at a dilution of 1 in 100 in 4 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at $A_{600}$ nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours or overnight before harvesting by centrifugation at 8000 g/10 min. Pellet from 1L of culture was stored at -80$^{o}$C.

**(c) Isolation and purification of [SCR1-3(delN195-K196)]TNANKSLSSISCQT**

The methods are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736, subsequently modified as described in Example 18. Briefly, the cell pellet from 1L of culture from (b) was resuspended in buffer, sonicated and the inclusion bodies isolated by centrifugation. The inclusion bodies were resolubilised in 100 ml of fully reducing buffer and target protein purified on Macroprep High S (30g wet weight). Product (27ml at nominal 1.5mg/ml) that eluted from the column in the 1M NaCl-containing buffer was refolded by dilution into 2.5L cold 60mM ethanolamine/1mM EDTA, with the glutathione redox agents added at 1h. After 3d at 4 degrees C the solution was ultrafiltered using a YM10 membrane and the retentate was treated with ammonium sulphate, centrifuged and the supernatant purified on Butyl Toyopearl 650M (bed volume 53ml). A single A280 peak was eluted by the decreasing ammonium sulphate gradient. SDS PAGE under non-reducing conditions followed by protein staining revealed a major polypeptide with an apparent molecular weight of 22 000, believed to be the target protein. One of the contaminating polypeptides had an apparent molecular weight of about 40 000, which was identified as the dimer of the monomeric form of the target by comparison with adjacent markers of [SCR1-3]-Cys. The product had an estimated protein concentration of 30uM.

**Example 20 Preparation of [SCR1-3(delN195-K196)]TNANKSLSSISC-(-S-S-[MSWP-1])QT (SEQ ID No. 17)**




[SCR1-3(delN195-K196)]TNANKSLSSISCQT prepared as described in Example 19 (approx. 30uM protein; 0.1ml) was mixed with TCEP (5mM in 50mM Hepes pH 4.5; 0.0072ml) and incubated at room temperature (22 degrees C) for 15h. 0.05ml of this solution was mixed with 0.005ml of 0.5M ethanolamine and 0.003ml of 7mM MSWP-1 (see Example 2); the solution was incubated for a further 4h at room temperature. SDS PAGE analysis showed a major band in the preparation had an apparent molecular weight

**Example 21 Preparation of [SCR1-3]DGPSEILRGDFSSC (SEQ ID No. 23)**

**(a) Construction of plasmid pBC04-31 encoding [SCR1-3]DGPSEILRGDFSSC**

Plasmid pBC04-31 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 25 and SEQ ID No. 26). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to >90oC and slowly cooling to room temperature and ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for the presence of a novel AvaI site at position 2733. On digestion with AvaI pBC04-31 yielded fragments of 2311 and 495bp. DNA from positive clones was used to transform the expression strains. The oligonucleotides inserted added the peptide sequence DGPSEILRGDFSSC to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

**(b) Expression, isolation and purification of [SCR1-3]DGPSEILRGDFSSC**

Expression, isolation and purification of **[SCR1-3]DGPSEILRGDFSSC** is carried out using pBC04-31 by procedures generally described in Example 6.

**Example 22 Preparation of [SCR1-3]DGPSEILRGDFSSC-(-S-S-[MSWP-1]) (SEQ ID No. 24)**

**$H_2N$-[SCR 1-3]-Asp-Gly-Pro-Ser-Glu-Ile-Leu-Arg-Gly Asp-Phe-Ser-Ser-Cys-OH**
**|**
**S**
**|**
**S**
**|**
**N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$**

[SCR1-3] DGPSEILRGDFSSC protein prepared in a similar way to that described in Example 21 is reacted with MSWP-1 as described in Example 8 to give the title compound.

**Example 23 Preparation of [SCR1-3] AAPSVIGFRILLLKVAGC (SEQ ID No. 33)**

**(a) Construction of plasmid pBC04-31 encoding [SCR1-3]**

Plasmid pBC04-34 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 34 and SEQ ID No. 35). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to >90°C and slowly cooling to room temperature and were ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for an increase in size of the Nde1/HindIII fragment by 59 base pairs. The presence of the cysteine codon was determined by the presence of a DdeI site at position 2781. pBC04-34 digested with DdeI yielded diagnostic bands of 481 and 109bp DNA from positive clones was used to transform the expression strains (see next section). The oligonucleotides inserted added the peptide sequence AAPSVIGFRILLLKVAGC to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

**(b) Expression,isolation and purfication of [SCR1-3]APSVIGFRILLLKVAGC**

Expression isolation and purification of [SCR1-3] APSVIGFRILLLKVAGC is carried out using pBC04-34 by procedures generally described in Example 6.

**Example 24 Preparation of [SCR1-3]AAPSVIGFRILLLKVAGC -(-S-S-[MSWP-1]) (SEQ ID No. 36)**

**$H_2N$-[SCR 1-3]-Ala-Ala-Pro-Ser-Val-Ile-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val-Arg-Gly-Cys-OH**

**|**

**S**

**|**

**S**

**|**

**N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-$NH_2$**

[SCR1-3]AAPSVIGFRILLLKVAGC protein prepared in a similar way to that described in Example 23 is reacted with MSWP-1 as described in Example 8.

**Biological Activity**

**(I) Anti-complement Activity Measured by the Classical Pathway-mediated Haemolysis of Sheep Erythrocytes**

(i) Functional activity of complement inhibitors was assessed by measuring the inhibition of complement-mediated lysis of sheep erythrocytes sensitised with rabbit antibodies (Diamedix Corporation, Miami, USA). The assay is designed to be specific for the classical pathway of complement activation. Human serum diluted 1:500 or 1:400 (final concentration in assay mixture) in 0.1 M Hepes/0.15 M NaCl/0.1% gelatin pH 7.4 was used as a source of complement. The serum was prepared from a pool of volunteers essentially as described in Dacie & Lewis, 1975. Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C or -80°C. Aliquots were thawed as required and diluted in the Hepes buffer immediately before use.

Inhibition of complement-mediated lysis of sensitised sheep erythrocytes was measured using a standard haemolytic assay using a v-bottom microtitre plate format as follows:

50 µl of a range of concentrations of inhibitor diluted in Hepes buffer were mixed with 50 µl of the diluted serum and 100 µl of sensitised sheep erythrocytes and then incubated for 1 hour at 37°C. Samples were spun at 1600rpm at ambient temperature for 3 minutes before transferring 150 µl of supernatant to a flat bottom microtitre plate and determining the absorption at 405 or 410 nm. Maximum lysis (Amax) was determined by incubating serum with erythrocytes in the absence of any inhibitor. Background lysis (Ao) was determined by incubating erythrocytes in the absence of any serum or inhibitor. Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis.

$$\% \text{ inhibition} = 1 - [(A - Ao) / (Amax - Ao)]$$

Results

| Compound | IH50 |
|---|---|
| WO94/00571<br>SEQ ID NO 27 | 0.2 - 0.3 ug/ml [10-15 nM] (1) |
| Example 6* | 0.65 ug/ml [30nM] (mean of two) (2) |
| Example 7* | 0.3-1.0 ug/ml [15-50nM] (n = 3) |
| Example 8a<br>Example 8b<br>Example 8c<br>Example 8d+ | 0.014 ug/ml [ 0.6 nM] )<br><0.001 ug/ml [< 0.04nM] ) (3)<br>0.001 ug/ml [0.043 nM] )<br>[0.06nM] ) |
| Example 10a<br>Example 10b<br>Example 12<br>Example 13<br>Example 14 | 0.02 ug/ml [0.8nM]<br>~0.01 ug/ml [~0.4nM]<br>~0.0016 ug/ml [0.07 nM]<br>~0.009 ug/ml [0.4nM]<br>~1.1 ug/ml [50nM] |
| Example 19 | [4nM] |

* As 2-mercaptoethanol/glutathione derivatives

+ Assay of the two solutions and the original pre-lyophilisation solution from Example 8d.

Other $IH_{50}$ values generated for similar batches include:

(1) 15nM

(2) 8nM, 5nM, 8nM, 4nM

(3) 0.3nM, 0.2nM, 0.07nM, 0.06nM, 0.2nM, 0.4nM, 0.5nM, 0.6nM.

The above data show that:

1. The complement inhibitory activities of the 'base' protein (SCR1-3 of human complement receptor 1 of WO94/00571) and its derivatives with either an additional C-terminal cysteine (SCR1-3/cys, Example 6) or a single cationic 'switch' sequence (SCR1-3/switch, Example 7) are similar.

2. However, incorporation of two membrane binding elements (electrostatic switch and myristoyl) by addition of MSWPs-1, 2 or 3 (which contain both elements) to SCR1-3/cys or three membrane binding elements by addition of the MSWP-1 to the SCR1-3/switch construct results in products which are significantly more potent (~20-200x) than the base or single membrane binding element proteins. The use of TCTP-1 which is

membrane addresses. Thus, the increases in potency in an assay which depends on an erythrocyte membrane event (cytolysis by the membrane attack complex of complement) can be attributed to membrane targeting of the cytolysis inhibitor proteins by the combination of two membrane binding elements.

**(ii) Assay of anti-complement activity in the classical pathway haemolytic assay: activity in the sera of domestic pig, guinea pig, rat and marmoset.**

The activity of [SCR1-3]-Cys-S-S-[MSWP-1] was examined in the classical pathway haemolytic assay using the sera of pig, guinea pig, rat or marmoset. The methodology was essentially as described in (I) with minor modifications, for example small changes to the concentration of serum used. [SCR1-3]-Cys-S-S-[MSWP-1] was prepared essentially as described in Example 8c. The IH50 values for the different sera were: pig, 0.2nM; guinea pig, 0.3nM; rat, 0.4nM; marmoset, 0.2nM. These results show that [SCR1-3]-Cys-S-S-[MSWP-1] is capable of inhibiting classical pathway complement inhibition in the sera of a variety of animal species.

**(II) Anti-complement Activity Measured by Alternative Pathway-mediated Haemolysis of Guinea Pig Erythrocytes**

Functional activity of complement inhibitors was assessed by measuring the inhibition of complement mediated lysis of guinea pig erythrocytes essentially as described by Scesney, S. M. et al (1996) J. Immunol. 26 1729-1735. The assay is designed to be specific for the alternative pathway of complement activation. Human serum prepared from a pool of volunteers essentially as described in Dacie & Lewis, 1975 was used as the source of complement. Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C or -80°C. Aliquots were thawed as required and diluted in 0.1 M Hepes/ 0.15 M NaCl / 0.1% gelatin / 8 mM EGTA / 5 mM $MgCl_2$ pH 7.4 (buffer A) immediately before use. Guinea pig erythrocytes were prepared from guinea pig whole blood collected into EDTA-coated tubes as follows. The blood was spun at 1600 rpm for 5 min and the erythrocyte pellet washed 3 times with 0.1 M Hepes/ 0.15 M NaCl / 0.1% gelatin pH 7.4 until the supernatant of the spin was essentially colourless. The erythrocytes were finally resuspended to the original volume of blood used and were stored at + 4 degrees C. They were used within 2 weeks.

50 µl of a range of concentrations of inhibitor diluted in buffer A in a v-bottom [illegible] were mixed with first 100 µl of serum that had been diluted 1:3 and

hour at 37°C. The plate was spun at 1600 rpm for 3 minutes before transferring 150 µl of each supernatant to a flat bottom microtitre plate and determining the absorption at 405 nm, which reflects the amount of lysis in each test solution. Maximum lysis (Amax) was determined by incubating serum with erythrocytes in the absence of any inhibitor. Background lysis (Ao) was determined by incubating erythrocytes in the absence of any serum or inhibitor. The final dilution of serum used in the assay did absorb at 405nm but the level of absorbance (approx 10% of Amax) was considered to have a neglible affect on the overall assay results and it was ignored in the calculations. Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis.

$$\% \text{ inhibition} = 1 - [(A - Ao)/(Amax - Ao)]$$

Results

Two aliquots (one lyophilised and resolubilised in a neutral buffer, the other not lyophilised) of a single batch of of [SCR1-3]-Cys-S-S-[MSWP-1] prepared in a similar way to that described in Example 8 (c) were tested in the haemolytic assay. The IH50 values for the compounds were:

| | |
|---|---|
| [SCR1-3]-Cys-S-S-[MSWP-1] (not lyoph) | 310nM |
| [SCR1-3]-Cys-S-S-[MSWP-1] (lyoph) | 480nM |

The result shows that [SCR1-3]-Cys-S-S-[MSWP-1] exhibited activity against the alternative pathway of the complement system and that lyophilisation and subsequent resolubilisation of the protein had no affect (within experimental error) on the biological activity of the protein.

**(III) Plasminogen activator assay**

**(i)** SK-related molecules from Example 16 were assayed using a plasminogen activation assay. A solution of purified human $Lys_{77}$-Plasminogen (1uM in PST buffer containing 25%v/v glycerol [PSTG buffer], 0.5ml) was incubated with thiolated SK (final concentration 0.1 to 1.07 nM) for 1h at 25°C. An aliquot of this mixture (10ul) was incubated with 1.0mM of the plasmin substrate S-2251 (H-D-Val-Leu-Lys-p-nitroanilide, KabiVitrum, Stockholm, Sweden) in 0.1M Triethanolamine HCl pH 8.0 (0.5ml) at 25°C. The release of p-nitroaniline was monitored continously at 405nm. Under these conditions, one substrate unit (SU) of plasmin activity is defined as the amount of enzyme giving an increase in optical density at 405nm of 0.001 $min^{-1}$. Under [illegible] nM [illegible] plasmin at a nearly linear of 4225 SU/ml

SK-MSWP-1 conjugate was diluted 1:100 in PSTG buffer and 5-50ul aliquots tested in the plasminogen activation assay. The stock preparation was found to contain approximately 2.9uM functional SK.

(ii) The potential activity of the acyl-enzyme preparations of Example 17 was estimated by dilution 25-50 fold into PST buffer and incubation for 2h at 37°C, followed by assay using 2mM S-2288 (H-D-Ile-Pro-Arg-p-nitroanilide 2HCl) under the same conditions used in (i) above. Under these conditions, the potential activity of the reduced PDAEB->tPA preparation was 2760 SU/ml and that of the MSWP-1/PDAEB->tPA conjugate 535 SU/ml.

**(IV) Erythrocyte binding assays**

**(i) Erythrocyte aggregation test for modified and unmodifed Rabbit anti-(human erythrocyte membrane) antibody.**

Human pooled erythrocytes (Ortho A2, Raritan, New Jersey, 3% v/v, 50ul) were added to microtiire plates wells and either unmodified rabbit anti-(human erythrocyte membrane) antibody [RAEM] or RAEM-MSWP1 conjugate from Example 15 added at concentrations expressed relative to undiluted stock RAEM. Cells were agitated at ~100rpm for 40min at 25°C. 5ul was removed from each well and examined by light microscopy at x 20 magnification. A visual scoring scale was used as follows:

- \- No clumping, cells moving freely relative to each other.
- \+ Small clumps (<10 cells)
- ++ Larger clumps (100 plus cells)
- +++Very large visible aggregates

Results

| | | | |
|---|---|---|---|
| Controls (n=6) | - | RAEM-MSWP1 1/3900 | +/- |
| RAEM 1/1100 | - | RAEM-MSWP1 1/1000 | +/- |
| RAEM 1/600 | - | | |
| RAEM 1/350 | +/- | RAEM-MSWP1 1/357 | +++ |
| RAEM 1/50 | ++ | RAEM-MSWP1 1/62 | +++ |

Conclusion

The antibody preparation modified to contain a membrane-binding unit was more effective at inducing aggregation of cells because binding to the cell membrane through MSWP1 allowed a higher effective concentration of bridging antibody on the membrane

**(ii) Binding of 125-Iodine-[SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocytes**

[SCR1-3]-Cys-S-S-[MSWP-1] (2mg/ml in PBS; 0.25ml) was mixed with 0.5mCi of 125-Iodine (Amersham) in the presence of 9nmoles potassium iodide following the Iodogen procedure and reagents (Pierce and Warriner (UK) Ltd.). The labelling was allowed to proceed for 20 min at room temperature, the reaction was quenched with 0.1ml of 1M potassium iodide and the solution buffer-exchanged into PBS/0.1% albumin. Citrated blood collected from a healthy volunteer was used as a source of human erythrocytes. Blood (0.2ml) was mixed with 10 microlitres of appropriately diluted 125-Iodine-[SCR1-3]-Cys-S-S-[MSWP-1] (final concentration 700pM) and incubated for 30min at 37 degrees C. The erythrocytes were then isolated by three repeat washings in PBS / centrifugation steps and samples counted in a Wallac 1470 Wizard gamma counter. The results were as follows:

| | cpm |
|---|---|
| 1st wash` | 3 600 000 |
| 1st pellet | 140 000 |
| 2nd wash | 52 000 |
| 3rd wash | 6 500 |
| final pellet | 26 000 |

Using values of 5 X $10^9$ erythrocytes per ml of blood and and a specific radioactivity of 2.7 X $10^7$ cpm / nmole for the [SCR1-3]-Cys-S-S-[MSWP-1] it was calculated that about 600 molecules of [SCR1-3]-Cys-S-S-[MSWP-1] bound per cell (the value for 'final pellet').

**(iii) Binding of fluorescein-labelled-[SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocytes**

[SCR1-3]-cys (prepared in a similar way to that described in Example 18) (45uM,1.0mg/ml in 0.1M sodium phosphate, approx. 0.2M ammonium sulphate pH 7.0 ) was partially reduced by incubation at 25°C for 18h by the addition of a 4-molar excess of Tris(2-carboxyethyl)phosphine (TCEP; Pierce & Warriner (UK) Ltd.). The solution was buffer exchanged into 50mM Hepes pH7.0; post buffer exchange the protein concentration was 22uM. The partially reduced [SCR1-3]-cys was incubated with a 4-fold molar excess of 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (Molecular Probes Inc., USA) and incubated for 1h at 4°C. The excess fluorescent label was removed by buffer exchange of the protein solution into 50mM Hepes pH7.0. Fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] was synthesised by adding MSWP-1 Example 2) to give a five fold molar excess over fluorescein labelled protein and was

incubated for 4h at 25°C. The solution was buffered exchanged into PBS and this solution was used for the microscopy studies.

[SCR1-3],10mg/ml in 50mM formic acid, was mixed in a 1:10 ratio with 50mM $NaHCO_3$ pH8.5; the pH of the solution was adjusted with NaOH to pH9.5. The fluorescein was extracted from Celite-fluorescein isothiocyante (Celite:fluorescein; 1:10, Sigma) by DMSO in a 1:4 (w/v) ratio. The fluorescein-DMSO solution was added to the protein solution in a 1:14 ratio and incubated for 1h at RT. Excess label was removed by gel filteration into PBS containing 0.01% Tween-80 and this solution was used for microscopy studies.

Citrated blood was collected from a healthy volunteer and the erythrocytes isolated, washed in PBS and diluted 250-fold compared to the original blood volume. 0.05ml of erythrocytes were incubated with 2uM fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] or 2uM fluorescein-[SCR1-3] and incubated for 30min at 37°C. An eight microlitre sample of each incubation was mounted on a slide and viewed on an inverted confocal microscope (Biorad). The cells incubated with fluorescein-[SCR1-3] showed no specific staining whereas with those incubated with fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] staining appeared diffusely over the cell surface and also intensely stained patches were visible on the cell membrane. No labelling was seen intracellularly.

**(iv) Binding of MSWP-1/PDAEB->tPA to human erythrocytes**

Human trypsinized and glutaraldehyde-treated red blood cells (1.0ml of a 4% suspension) was pelleted by low-speed centrifugation and resuspended in a total volume of 0.5ml PST containing either no additions or approximately 270 SU of either reduced PDEAB->tPA or MSWP-1/ PDAEB->tPA conjugate of Example 17. The mixtures were incubated by gentle rolling for 5 min at 23°C and then the cells were pelleted by centrifugation followed by two washes with 1.0ml PST buffer. Finally, the cells were suspended in 0.5ml PST and incubated at 37°C . Samples of the supernatant (100ul) were removed after pelleting. Assay using S-2288 (as above ) showed that after 2h, approximately 7% of the applied t-PA activity was present in the supernatant of cells exposed to MSWP-1/PDAEB->tPA whereas only~2.8% was present in the supernatant of cells exposed to reduced PDAEB->tPA alone. No t-PA amidolytic activity was detected in controls.

This experiment suggests that reversible linkage of the active site of t-PA to MSWP-1 increases the tendency of this enzyme to bind to red blood cells.

**(v) Localisation of SK-MSWP-1 conjugate on the surface of human erythrocytes**

A stabilised preparation of human erythrocytes (trypsinised, glutaraldehyde-treated, Sigma, Gillingham,UK, 4% v/v, 0.4ml) was pelleted by centrifugation (~2000g/2min) and resuspended in 0.4ml PST buffer with either 0.1uM thiolated SK or 0.1uM SK-MSWP-1 from Example 16.

The suspensions were incubated for 30 min at 37°C and then washed by two cycles of centrifugation and resuspension in PST buffer. Finally, they were resuspended in PSTG buffer (0.4ml) containing 1uM plasminogen and incubated and assayed for plasmin as described above.

The control thiolated-SK generated plasmin at a rate of 522 SU/ml, while the SK-MSWP-1 conjugate produced 6184 SU/ml.The latter activity corresponds to around 2100 thiolated SK molecules/cell.

**(vi) Binding of [SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocyte membranes**

4 X 2.0ml of trypsinized, glutaraldehyde-treated human erythrocytes (Sigma, R0127) were centrifuged for 2min at about 3000 rpm. The supernatants were discarded and the cells resuspended in phosphate/saline/Tween (0.01%) (PST) (1ml per tube) and [SCR1-3]-Cys-S-S-[MSWP-1] of Example 8 was added to a final concentration of 20ug/ml to three of the tubes. The mixtures were then incubated at 37 degrees C for 30 min., then washed five times by repeat centrifugation and washing in PST. The cells were finally suspended in 1ml PST and were held at 4 degrees C.

The ability of these cells to inhibit complement-mediated lysis of sheep erythrocytes was measured using the standard classical pathway complement inhibition assay described in (I) above. The human erythrocytes were added to the assay at four different dilutions, followed by the human serum and then the sheep red blood cells and incubation at 37 degrees C as usual. The % inhibition data are shown below.

| Final dilution | human erythrocytes, untreated | human erythrocytes, treated with [SCR1-3]-Cys-S-S-[MSWP1] |
|---|---|---|
| 1/4 | 22% | 62% |
| 1/16 | -8% | 88% |
| 1/256 | 5% | 74% |
| 1/2500 | -7% | 51% |

Thus the percentage inhibition for the [SCR1-3]-Cys-S-S-[MSWP-1]-treated cells at maximum dilution was significantly higher than the untreated cells at 1/4 dilution. The [SCR1-3]-Cys-S-S-[MSWP-1]-treated cells, therefore, contained at least 600-fold [illegible] inhibitory activity than the untreated cells, even though the cells had